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(54) Title: BIOSENSOR FOR AND METHOD OF ELECTROGENERATED CHEMILUMINESCENT DETECTION OF NUCLEIC ACID ADSORBED TO A SOLID SURFACE

(57) Abstract

    Single-strand DNA was immobilized on an electrode covered with an aluminum alkanebisphosphonate film by immersing it in an ss-DNA solution. The immobilized ss-DNA labeled with $Ru(bpy)_3^{2+}$ was detected by monitoring the electrogenerated chemiluminescence (ECL) produced upon oxidation in a solution containing tri-n-propylamine. After immobilization of unlabeled ss-DNA, a complementary labeled strand DNA was hybridized to produce ds-DNA on the surface. The extent of DNA hybridization was determined by ECL of the labeled $Ru(bpy)_3^{2+}$. Surface immobilized ds-DNA could also be detected by observing the ECL of intercalated $Ru(phen)_3^{2+}$. Transmission electron microscopy (TEM) was employed to image the film and the immobilized DNA.

# Title of the Invention

## BIOSENSOR FOR AND METHOD OF ELECTROGENERATED CHEMILUMINESCENT DETECTION OF NUCLEIC ACID ADSORBED TO A SOLID SURFACE

5      ## Field Of Invention

The present invention relates to the diagnostic field, and especially nucleic acid diagnostics. In particular, the present invention relates to a probe or sensor having a film containing metal centers, its preparation where

10     a single-strand or double-strand nucleic acid sequence is immobilized thereon on, and its use in the subsequent detection of the nucleic acid by labelling with luminescent metal chelates.

In addition to using the subject biosensor for

15     nucleic acid diagnostics based on a surface designed modified electrical sensor, i.e., chips or electrode, immobilization and hybridization of nucleic acid such as DNA on a self-assembled thin film via surface reaction is also useful in studying molecular recognition of DNA.

20     ## Background Of Invention

Nucleic acid diagnostics has become an important area in molecular biology and biotechnology studies, with applications to the determinations of disease and food contaminating organisms and in forensic and environmental

25     investigations. The development of new DNA biosensors has led to the application of several detection techniques such as

optical methods (e.g., luminescence, ellipsometry and pseudo-Brewster angle reflectometry), piezoelectric devices (e.g., SAW, QCM), and electrochemical techniques (e.g., CV and SWV). Of particular interest are labels which can be made to

5    luminesce through photochemical, chemical, and electrochemical reaction schemes. In particular, electrochemiluminescent methods of determining the presence of labelled materials are preferred over other methods for many reasons. They are highly diagnostic of the presence of a particular label,

10   sensitive, nonhazardous, inexpensive and can be used in a wide variety of applications. Suitable labels comprise electrochemiluminescent compounds, including organic compounds and metal chelates.

For example, electrochemiluminescent ruthenium- and

15   osmium-containing labels have been used in methods for detecting and quantifying analytes of interest in liquid media (U.S. Patent Nos. 5,310,687; 5,238,808; and 5,221,605). In addition, the application of electrogenerated chemi-luminescence (ECL) measurements to the detection of solution

20   phase DNA intercalated with ruthenium-containing labels has been described (Carter, M.T. et al. (1990) Bioconjugate Chem 2:257-263). However, reaction schemes that are viable in the solution phase are often not applicable in the solid phase. More importantly, the detection of solution phase analytes

25   such as DNA has several drawbacks relative to detection of analytes absorbed to solid surfaces. The advantages for detecting DNA via solid phase techniques as opposed to

solution t chniques are:  (1) more sensitive (detection of
monolayer quantities); (2) easier to separate DNA from sample
(avoid interferences); and (3) possibility of detection of
several different DNA in single analysis, with localized

5      probes, e.g., as in sequencing studies.

These luminescent systems are of increasing
importance in diagnostics.  For example, in U.S. Pat. No.
4,372,745, chemiluminescent labels are used in immunochemical
applications where the labels are excited into a luminescent

10     state by reaction of the label with $H_2O_2$ and an oxalate.  In
these systems, $H_2O_2$ oxidatively converts the oxalate into a
high energy derivative, which then excites the label.  It is
expected, that in principle, the $H_2O_2$ and an oxalate reaction
scheme should work with any luminescent material that is

15     stable under the oxidizing conditions of the assay, and can be
excited by the high energy oxalate derivative.  Unfortunately,
this very versatility is a major drawback of the 4,372,745
patent:  lack of selectivity or specificity, i.e., typical
biological fluids containing the analyte of interest also

20     contain a large number of potentially luminescent substances
that can cause high background levels of luminescence.

Thus a need exists for a solid phase system, e.g., a
biosensor, and method that (1) provides the necessary
specificity absent systems that rely on the $H_2O_2$ and oxalate

25     reaction scheme; and (2) do not depend on solution techniques.
The present invention overcomes the limitations and drawbacks

of the prior art.


## Summary Of Invention

The present invention provides a biosensor and its use for electrogenerated chemiluminescent detection of nucleic acid absorbed to a solid surface via the use of ruthenium- and osmium-containing chemiluminescent labels.

An object of the present invention is to provide a film containing an aluminum (III) alkanebisphosphonate layer having metallic aluminum centers, i.e., ionic aluminum Al(III) centers, for bonding to single-strand or double-strand DNA immobilized to said aluminum centers. The aluminum (III) alkanebisphosphonate can be provided as a biosensor having a coating of $Al_2(C_4BP)$ to bond to SS-DNA or ds-DNA.

A further object of the present invention is to provide a biosensor in the form of chips or electrodes with adsorbed DNA that is labelled with a luminescent label, such as an osmium or ruthenium moiety.

A still further object of the present invention is to prepare a biosensor by treating a silicon wafer to form a chromium layer and juxtaposed gold layer, then contacting the layered wafer with an anchoring agent; and subsequently immersing the product in $Al(NO_3)_3$, bisphosphonic acid $(H_2O_3P(CH_2)_4PO_3H_2)$ and $Al(NO_3)_3$ aqueous solutions.

Another object of the present invention involves the detection of a nucleic acid by labelling with luminescent metal chelates.

        A further object of the present invention is to
apply electrogenerated chemiluminescent techniques to a
plurality, i.e., arrayed, oligonucleotide probes.

        These and other objects will become more apparent
5   from the following detailed description and drawings.


Brief Description Of The Drawings

        Fig. 1    shows a schematic representation of the
                  silicon electrode of the present invention
                  containing ionic aluminum Al(III) sites.

10      Fig. 2    shows immobilization of ds-DNA on a
                  $Al_2(C_4BP)$ film and interaction of
                  $Ru(phen)_3^{2+}$ with the ds-DNA.

        Figs. 3A-3C show first (A), second (B), and third
                  (C) scans.  ECL emission-potential
15                transients at the $Al_2(C_4BP)/DNA-Ru(phen)_3^{2+}$
                  electrode in 0.19 M phosphate buffer, pH
                  7, containing 0.13 M TPrA.  (Scanning was
                  halted and the solution stirred after each
                  scan.)  Scans were initiated at 0 V and
20                were directed toward more positive
                  potentials.  Scan rate, 50 mV/s.

        Figs. 4A-4C show schematic representations of
                  immobilization of ss-DNA tagged with
                  $Ru(bpy)_3^{2+}$ on the film (Fig. 4A);
25                immobilization of ss-DNA on the film and
                  hybridization of complementary strand DNA

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tagged with Ru(bpy)$_3^{2+}$ (Fig. 4B);

immobilization of poly(dA) on the film,

hybridization of poly(dT), and then

interaction of Ru(phen)$_3^{2+}$ with the ds-

5       DNA(poly(dA)∘poly(dT)) where the ∘

represents an ECL active species.

Figures 5A-5C show a cyclic voltammogram (Fig. 5A);

an emission-potential transient of the

Al$_2$(C$_4$BP)/λ-1 ss-DNA-Ru(bpy)$_3^{2+}$ electrode in

0.19 M phosphate buffer/0.13 M TPrA, pH 7

10      (Fig. 5B); and an emission-potential

transient of the Al$_2$(C$_4$BP)λ-1 ss-DNA in the

same solution (Fig. 5C). The electrode

used in the experiments shown in Figures

5A-5C was prepared as described in the

15      specification with immersion in a 1.38 μM

λ-1 ss-DNA-Ru(bpy)$_3^{2+}$ or λ-1 ss-DNA

solution for ~4 h, respectively. In each

case, the potential was scanned from 0 t

1.60 V at v = 50 mV/s.

20

Figure 6 shows an emission-time transient for the

Al$_2$(C$_4$BP)/λ-1 ss-DNA-Ru(bpy)$_3^{2+}$ electrode in

0.19 M phosphate buffer/0.13 M TPrA, pH 7,

when the potential was stepped from 0 to

25      1.5 V.

Figures 7A and 7B show ECL emission-potential

transi nts at the Al$_2$(C$_4$BP)/λ-1c ss-DNA/λ-1

ss-DNA-Ru(bpy)$_3^{2+}$ electrode (Fig. 7A) and
at the Al$_2$(C$_4$BP)/$\lambda$-1 ss-DNA/$\lambda$-1 ss-DNA-
Ru(bpy)$_3^{2+}$ electrode (Fig. 7B) where both
electrodes are immersed in 0.19 M
phosphate buffer, pH 7, containing 0.13 M
TPrA and the potential was scanned from 0
to 1.60 at scan rate, 50 Mv/s.

Figures 8A and 8B show ECL emission-potential
transients at the
Al$_2$(C$_4$BP)/poly(dA)/poly(dT)/Ru(phen)$_3^{2+}$
electrode (Fig. 8A) and at the
Al$_2$(C$_4$BP)/poly(dA)/Ru(phen)$_3^{2+}$ electrode
(Fig. 8B) where both electrodes are
immersed in 0.19 M phosphate buffer, pH 7,
containing 0.13 M TPrA, and the potential
was scanned from 0 to 1.60 at scan rate,
50 mV/s.

Figures 9A-9C show TEM images of Au substrate coated
on a Formvar film on a #400 Cu grid (Fig.
9A); the Al$_2$(C$_4$BP) film on the Au substrate
(Fig. 9B); immobilized calf thymus ds-DNA
on the Al$_2$(C$_4$BP) film, prepared by
immersing the film in a 1.65 mM [NP] of
ds-DNA solution for ~ 4 h (Fig. 9C).

Figures 10A-10C show TEM images of the Al$_2$(C$_4$BP)
film on Au substrate coated on a Formvar
film (Fig. 10A); immobilized calf thymus

ds-DNA on the $Al_2(C_4BP)$ film, prepared by
immersing the film in a 1.65 mM [NP] of
ds-DNA solution, for ~4 h (Fig. 10B);
immobilized sonicated calf thymus ds-DNA

5              on the $Al_2(C_4BP)$ film, prepared by
immersing the film in a 1.65 mM [NP] of
ds-DNA solution, sonicated for ~6 h at
room-temperature, for ~4 h.  (Fig. 10C)
         Figures 11A - 11B show sequencing via array

10                        hybridization.


Detailed Description Of Invention

         The present invention relates to a sensor and method
of detecting nucleic acids using the sensor.  The sensor can
be a chip, electrode, or an appropriately modified surface for
15  adsorbing ss-DNA or ds-DNA.  The nucleic acids detected by the
method of the present invention include DNA, cDNA or any
synthetic variant thereof.  A nucleic acid as used throughout
the specification and in the claims is meant DNA or any
synthetic variant thereof.

20        Examples of DNAs detectable by the present method
include chromosome DNA, plasmid DNA, viral DNA, bacterial DNA
and recombinant DNA.  The length of nucleic acid sequence
capable of detection by the present method ranges from about
2.7 nm to about 200 nm.  In a preferred embodiment, the
25  nucleic acid sequence rang s from 8 base pair (bp)
nucleotides to 3,000 base pair nucleotides.  In a most

preferr d embodiment ranges from about 30 bp nucleotides t
1,500 bp nucleotides.

5

10

In the present invention, the nucleic acid sequence
to be detected may be of purified nucleic acid or may be
present in a biological sample. Biological samples in which
nucleic acids can be detected using the method of the present
invention include but are not limited to biological fluids,
e.g., serum, saliva, hair, skin, etc. Alternatively, the
nucleic acid can be purified from a sample using methods known
to those skilled in the art (Current Protocols in
Electrobiology (1994 ed. Ausubel, F.M. et al. John Wiley &
Sons, Inc.)).

15

20

The aluminum (III) alkanebisphosphonate preferably
used is a $Al_2(C_4BP)$, also $[Al_2C_4BP]$, film bearing biosensor and
is prepared as follows. Silicon wafers were soaked in
trichloroethylene for 30 min, rinsed twice with 2-propanol,
rinsed with excess amount of deionized water, and then dried
with a stream of dry nitrogen. The clean silicon wafers were
primed with a 50 Å chromium layer followed by deposition of a
2000 Å gold layer. Chromium and gold targets (99.999%) were
used to sputter the films onto the wafers in a MRC Model 8620
system at $10^{-2}$ torr. Other techniques, such as chemical vapor
deposition (CVD) to apply the Au or Cr layers, can also be
used.

25

The gold surface supported on the silicon wafers was
cleaned with hot chromic acid (saturated $K_2Cr_2O_7$ in 90% $H_2SO_4$)
for ~10 s and then rinsed with copious amounts of water. This

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process was repeated until the surface contact angle with
water was less than 15°. The clean Au surface was then
immediately soaked in an anchoring agent, 0.5 mM 4-
mercaptobutylphosphonic acid (MBPA) solution in absolute

5      ethanol for ~24 h. See Fig. 1. The phosphoric acid
terminated surface was then thoroughly rinsed with the
ethanol, dried with a stream of $N_2$ and then immersed
alternately in 5 mM $Al(NO_3)_3$, 5 mM bisphosphonic acid
$(H_2O_3P(CH_2)_4PO_3H_2)$ and 5 mM $Al(NO_3)_3$ aqueous solution, taking ~4 h

10     for each immersion, with washing with water between each step.
Although the preferred embodiment contemplates the use of Al
centers, other metal centers, such as, lanthanum (La) and
zirconium (Zr) are also contemplated. In addition, although a
$C_4$ specie was utilized in producing the alkanebisphosphonate

15     film, other chain links, $C_2$-$C_{16}$ may be utilized, which result in
adequate spacing of the aluminum ions on the surface of the
film to permit contact with the phosphates of the DNA
backbone. In other words, the "spacer" may range from 2 to 16
carbons in length.

20             The sensor described above is used by:

         a)     adsorbing a nucleic acid onto a film containing
metal centers;

         b)     reacting nucleic acid adsorbed to said film
with a luminescent metal label; and

25             c)     detecting the nucleic acid metal label chelates
formed in step b) via electrogenerated chemiluminescence of
said chelates.

In step (a), th  film to which the nucleic acid is adsorbed should contain metal ions which are suitably spaced on the surface of the film to allow interaction of the metal with the phosphate backbone of the nucleic acid sequence.

5    Examples of metal centers suitable for use in binding to nucleic acid phosphate groups are aluminum, lanthanum, and zirconium.  In a preferred embodiment, the film contains an aluminum Al (III) metal center.

The nucleic acid adsorbed to the film in step (a) of

10   the method of the present invention may be either double-stranded or single-stranded.  When a single-stranded nucleic acid is adsorbed to the film, the adsorbed single-stranded nucleic acid is then hybridized to a complementary single-stranded nucleic acid sequence.  Conditions of hybridization

15   are utilized which promote base pairing between the single-stranded DNA adsorbed to the film in its complementary sequence.  Factors influencing hybridization between nucleic acid sequences are known to those skilled in the art and include salt concentration of the hybridization solution,

20   hybridization temperature and stringency of post-hybridization washes.  In addition, the length of hybridization may also be controlled to optimize binding.  Satiable buffers in which to carry out the hybridization reaction include 5mM Tris buffer, pH 7 containing 50 mM NaCl.

25   The complementary single-stranded nucleic acid sequence hybridized with the nucleic acid adsorbed to the film may be unlabeled or labeled with a luminescent metal label.

Suitabl luminescent labels include ruthenium- and osmium-
containing labels where ruthenium or osmium are bound to at
least one polydentate ligand. If the metal has greater than
one polydentate ligand, the polydentate ligands may be the
5      same or different.  (Other known ECL active labels can also be
utilized that emit at different wave lengths such as organic
ECL labels, e.g. sulfonated-9,10-diphenylanthracene.)
Polydentate ligands of either ruthenium or osmium include
aromatic and aliphatic ligands.  Suitable aromatic polydentate
10     ligands include aromatic heterocyclic ligands.  Preferred
aromatic heterocyclic ligands are nitrogen-containing, such
as, for example, bipyridyl, bipyrazyl, terpyridyl, and
phenanthrolyl.  If the metal chelate has greater than one
polydentate ligand, the polydentate ligands may be the same or
15     different.

        Suitable polydentate ligands may be unsubstituted,
or substituted by any of a large number of substituents known
to the art.  Suitable substituents include for example, alkyl,
substituted alkyl, aryl, substituted aryl, aralkyl,
20     substituted aralkyl, carboxylate, carboxaldehyde, carboxamide,
cyano, amino, hydroxy, imino, hydroxycarbonyl, aminocarbonyl,
amidine, guanidinium, ureide, sulfur-containing groups,
phosphorous containing groups, and the carboxylate ester of N-
hydroxysuccinimide.

25     The ruthenium or osmium may have one or more
monodentate ligands, a wide variety of which are known to the
art.  Suitable monodentate ligands include, for example,

carbon monoxide, cyanides, isocyanides, halides, and
aliphatic, aromatic and heterocyclic phosphines, amines,
stibines, and arsines. A more complete list of the ligands,
e.g., monodentate and polydentate ligands, that can be used in
5      the present invention are set forth in U.S. Patent Nos.
5,310,687, 5,238,808 and 5,221,605, the subject matter of
which are incorporated herein by reference.

It is also within the scope of this invention for
one or more of the ligands of the metal to be attached to
10     additicnal chemical labels, such as, for example, radioactive
isotopes, fluorescent compounds, or additional luminescent
ruthenium- or osmium-containing centers.

The complementary single-stranded nucleic acid may
be tagged with the preferred luminescent metal labels of the
15     present invention via co-valent bonding to one or more of the
polydentate ligands of the metal label through one more amide
linkages. This linkage may be oriented so that the nucleic
acid is bonded directly either to the carbinol or to the
nitrogen of the amide linkage. These chemical moieties may be
20     ionized. A more elaborate description of methods for
attaching a luminescent ruthenium-containing or osmium-
containing label to amino groups of biological substances such
as nucleic acid is provided in U.S. Patent No. 5,221,605
incorporated by reference.

25     In an alternative embodiment, the complementary
single-stranded nucleic acid is unlabeled and hence,
hybridization to a single-stranded nucleic acid adsorbed to a

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film results in generation of unlabeled double-stranded

nucleic acid adsorbed to the film.  Thus, double-stranded

nucleic acid can be adsorbed to the film directly or can be

created by first adsorbing single-stranded nucleic acid to the

5      film and then hybridizing the adsorbed nucleic acid with its

complementary sequence.  In either case, the film containing

the adsorbed double-stranded nucleic acid is then immersed in

a solution containing luminescent metal label or a solution

suitable for promoting intercalation of the metal with the

10     double-stranded nucleic acid.  Examples of suitable solutions

include, but are not limited to water.

A nucleic acid in which the luminescent metal

containing labels intercalates to produce nucleic acid--metal

label chelates is then detected by inducing the metal label

15     present in the chelates to emit electromagnetic radiation by

creating an excited state of the metal species that will

luminesce at wave lengths from about 200 nanometers to about

900 nanometers, at ambient temperatures.  Intercalation (or

more generally, association) of the ECL labeled species with

20     DNA depends upon the experimental conditions in which the

label is partially inserted between the base pairs of DNA.  It

is considered "association" because of an electrostatic

interaction between a positively-charged label and the

negatively-charged phosphate groups on the DNA.  The exact

25     nature of the interaction of $Ru(phen)_3^{2+}$ with DNA is uncertain,

but is believed to be intercalation.  The temperature must be

b low the melting point of ds-DNA, preferably about 25-30° C.

The pH is typically near 7, but within a range of about 5 to about 8. The intercalation or association reaction must be given sufficient time to occur; about 30 to about 60 minutes, although times as short as 10 minutes also work.

5        In one embodiment of the present invention, the metal label is excited by exposing the nucleic acid-metal label chelates to electrochemical energy. The potential at which the oxidation of the metal label will occur depends upon the exact structure of the metal label as well as factors such

10       as the co-reactant utilized, the pH of the solution and the nature of the electrode used. Examples of suitable co-reactants which, when incubated with the nucleic acid-metal label chelates in the presence of the electrochemical energy, will result in emission of the metal label intercalated with

15       the nucleic acid, include tripropylamine (TPrA), oxalate or other organic acid such as pyruvate, lactate, malonate, tartrate and citrate. This oxidation can also be performed chemically, with some strong oxidants such as $PbO_2$ or a Ce(IV) salt.

20       Those of ordinary skill in the art recognize how to determine the optimal potential and emission wave length of an electrochemiluminescent system. The electrochemiluminescent species may be measured by any suitable mechanism such as measurement of an electric current or emitted electromagnetic

25       radiation. For example, the rate of energy inputted into the system can provide a measure of the luminescent species. Suitable measurements include, for example, measurements of

electric current when the luminescent species is generated
electrochemically, the rate of reductant or oxidant
utilization when the luminescent species is generated
chemically or the absorption of electromagnetic energy in
5      photoluminescent techniques.  In addition, of course, the
luminescent species can be detected by measuring the emitted
electromagnetic radiation.  All of these measurements can be
made either as continuous, rate-based measurements, or as
cumulative methods which accumulate the signal over a long
10     period of time.  An example of rate-based measurements would
be by using photomultiplier tubes, photodiodes or
phototransistors to produce electric currents proportional in
magnitude to the incident light intensity.  Examples of
cumulative methods are the integration of rate-based data, and
15     the use of photographic film to provide cumulative data
directly.

        All of these luminescence-based methods entail
repeated luminescence by the ruthenium-containing compound.
The repetitive nature of the detectable event distinguishes
20     these labels from radioactive isotopes or bound
chemiluminescent molecules such as luminol.  The latter labels
produce a detectable event only once per molecule (or atom) of
label, thereby limiting their detectability.

        The following examples illustrate various aspects of
25     the invention but are in no way intended to limit the scope
thereof.  Analysis was performed in a Plexiglas cell designed
for ECL and electrochemical studies using the film of th

present invention, a gold surface supported on the silicon wafer containing metallic sites, e.g., Al. A saturated calomel reference electrode (SCE) and a platinum wire counter

5    electrode were used for all measurements. The electrochemical measurements coupled with ECL experiments were carried out with a Model 175 universal programmer, a Model 173 potentiostat (Princeton Applied Research, PAR, Princeton, NJ), and an Ominigraphic 2000 X-Y recorder (Houston Instruments,

10   Houston, TX). The ECL emission was detected by a Model C123 single-photon-counting system (Hamamatsu Corp., Bridgewater, NJ) utilizing a Hamamatsu R928P PMT, cooled to -20°C in a Model TE 308 TSRF cooler controller (Products for Research Inc., Danvers, MA). The meter output was fed into the y-axis

15   of the x-y recorder, and the signal from the potentiostat was fed into the x-axis to afford ECL intensity versus bias potential display. Solution analysis by ECL was carried out with a QPCR analyzer (Perkin-Elmer, Norwalk, CT).

A MRC (Materials Research Corporation, Orangeburg,

20   NY) Model 8620 sputtering system at $10^{-2}$ torr, with an RF power of 150 W and RF peak to peak voltage of 1.8 KV, was used to sputter gold (99.999%) on silicon wafers.

Polydeoxyadenylic acid (Poly(dA)), polythymidylic acid (poly(dT), polydeoxycytidylic acid (poly(dC)) and calf

25   thymus (CT) ss-DNA and ds-DNA were obtained from Sigma Chemical Co. (St. Louis, MO) and were used without additional purification. The ss-DNA samples, $\lambda$-1 DNA (5'GAAAATGTGCTGACCGGACATGAAAATGAG3'), (Seq. ID. No. 1) $\lambda$ DNA

tagged with $Ru(bpy)_3^{2+}$ ($5'Ru(bpy)_3^{2+}$-

GAAAATGTGCTGACCGGACATGAAAATGAG3'), (Seq. ID. No. 2) and a

complementary strand λ-lc DNA

5      (5'CTCATTTTCATGTCCGGTCAGCACATTTTC3'), were obtained from

Perkin-Elmer and diluted with a 5 mM tris buffer containing 50

mM NaCl(pH 7). Synthesis of ss-DNA can be carried out on a

DNA synthesizer (e.g. Applied Biosystems, Model 381A). See

also L.J. McBride and M.H. Cruthers, Tetrahedron Letters, 24,

10     245 (1983).

The reagents used in the following examples include

trichloroethylene (99.6%), 2-propanol (99.9%), tripropylamine

(TPrA) (98%), $Ru(bpy)_3Cl_2 \cdot 6H_2O$, $Ru(phen)_3Cl_2O$, ethyl alcohol (200

proof), $Al(NO_3)_3 \cdot 9H_2O$, $K_2Cr_2O_7$, $NaH_2PO_4$ and tris(hydroxymethyl)

15     aminomethane and were used as received without purification.

Bisphosphonic acid $H_2O_3P(CH_2)_4PO_3H_2$ ($C_4BPA$), and 4-

mercaptobutylphosphonic acid (MBPA) were synthesized in

accordance with the techniques taught by Mallouk et al,

J.A.C.S., 115, 11855 (1993). The assay buffer for ECL

20     experiments contained 0.13 M TPrA and 0.19 M phosphate buffer,

prepared by dissolving TPrA into a $NaH_2PO_4$ solution and

adjusting the pH to 7 with 1 M NaOH.

Deionized water from a Millipore Milli-Q (18 MΩ-cm)

system was used to prepare all aqueous solutions and to rinse

25     the electrode surface.


TEM Sample Preparation.

TEM samples were prepared by coating Au on a Formvar
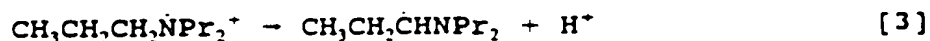
film on a #400 Cu grid with a vacum evaporator (Edwards 306),

fabricating the $Al_2(C_4BP)$ film on Au following the procedure described above and then immobilizing DNA on the $Al_2(C_4BP)$ film.  A transmission electron microscope (JEOL 100CX) at 80 KV was used to image the Au substrate, the $Al_2(C_4BP)$ film and

5     the immobilized DNA.
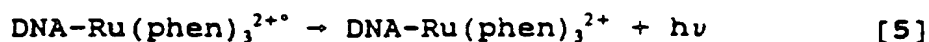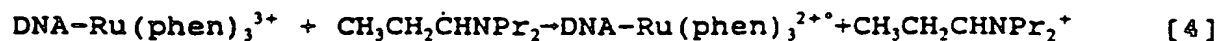
<u>Example 1</u>

<u>Electrogenerated Chemiluminescent Detection of</u>

<u>Immobilized ds-DNA using A Ruthenium Label</u>.

Calf thymus ds-DNA was immobilized on the surface of

5      an Al$_2$(C$_4$BP) film by immersing the film in a solution of DNA

(1.9 mM in nucleotide phosphate, NP) for 4 h (Figs. 1 and 2).

The film was then rinsed three times with 4-mL portions of

deionized water and then immersed for 4 h in either an aqueous

0.56 mM Ru(phen)$_3$Cl$_2$ solution or a 0.12 mM Ru(phen)$_3$(ClO$_4$)$_2$

10     solution in MeCN.  Ru(phen)$_3^{2+}$ associates with ds-DNA and can

be detected through its electrogenerated chemiluminescence

(ECL).  Alternatively, the film could be soaked in a mixed ds-

DNA (1.9 mM NP) and Ru(phen)$_3$Cl$_2$ (0.12mM) solution for 4 h to

produce the adsorbed layer.

15          ECL was produced by scanning the potential of the

electrode following film formation, DNA adsorption, and

Ru(phen)$_3^{2+}$ association, from 0 to 1.6 V vs. a saturated

calomel electrode (SCE) while it was immersed in a solution of

0.19 M phosphate buffer (pH 7) containing 0.13 M tri-n-

20     propylamine (TPrA).  Typical ECL transients, detected with a

single-photon-counting apparatus, are shown in Figures 3A-3C.

Emission arises from the energetic electron-transfer reaction

between electrogenerated Ru(phen)$_3^{3+}$ and an intermediate in the

oxidation of TPrA:

25          DNA-Ru(phen)$_3^{2+}$ (ads) - e → DNA-Ru(phen)$_3^{3+}$     [1]

CH$_3$CH$_2$CH$_2$-N̈-Pr$_2$ - e → CH$_3$CH$_2$CH$_2$NPr$_2^+$          [2]

CH$_3$CH$_2$CH$_2$ṄPr$_2^+$ → CH$_3$CH$_2$ĊHNPr$_2$ + H$^+$          [3]

$$DNA-Ru(phen)_3^{3+} + CH_3CH_2\dot{C}HNPr_2 \rightarrow DNA-Ru(phen)_3^{2+\cdot}+CH_3CH_2CHNPr_2^+ \qquad [4]$$

$$DNA-Ru(phen)_3^{2+\cdot} \rightarrow DNA-Ru(phen)_3^{2+} + h\nu \qquad [5]$$

The emission intensity decreased on a second scan and none was
seen on a third, suggesting loss of $Ru(phen)_3^{3+}$ from the film
and diffusion into the bulk solution.

In a control experiment, an electrode with a film of
$Al_2(C_4BP)$ that had not been treated with DNA was soaked for 4 h
in either an aqueous 0.56 mM $Ru(phen)_3^{2+}$ or a 0.12 mM
$Ru(phen)_3^{2+}$ in MeCN solution and then rinsed with MeCN and
water.  This electrode showed no ECL emission upon potential
sweep in the same TPrA solution described above, demonstrating
the adsorption of $Ru(phen)_3^{2+}$ on the $Al_2(C_4BP)$ film does not
occur.  The ability to generate $Ru(phen)_3^{3+}$ electrochemically
in this ECL experiment demonstrates that the $Al_2(C_4BP)$ film and
the DNA layer do not prevent heterogeneous electron-transfer
reactions.

To obtain further evidence for $Al_2(C_4BP)$ film
formation and DNA immobilization, experiments were undertaken
with a gold-coated quartz crystal and a quartz crystal
microbalance (QCM).  The gold was repeatedly treated with hot
chromic acid and then rinsed with water and EtOH until the
surface was hydrophilic, as indicated by contact angle
measurements.  The crystal frequency was then measured in air
during different stages of $Al_2(C_4BP)$ film formation, after DNA
adsorption, and after $Ru(phen)_3^{2+}$ association.  The film was
rins d after each step with deionized water and dried in a

stream of $N_2$ before measurement of the frequency.  Results are shown in Table 1.

Table 1.   Frequency and Mass Changes on a QCM Plate £ r

Film Growth, DNA Immobilization, and Ru(phen)$_3^{2+}$ Binding.

| After immersion in: [a] | $\Delta f^g$ (Hz) | $\Delta m$ (ng) | $10^9 \Gamma^h$ (mol/cm$^2$) |
|---|---|---|---|
| MBPA [b] | -70 | 137 | 1 |
| Al(NO$_3$)$_3$ [c] | -27 | 53 | 1 [i] |
| C$_4$BPA [d] | -145 | 283 | 2 |
| Al(NO$_3$)$_3$ [c] | -25 | 49 | 1 [i] |
| ds-DNA [e] | -24 | 47 | 0.3 [j] |
| Ru(phen)$_3^{2+}$ [f] | -18 | 35 | 0.1 |

[a] Immersions were sequential in order given from top to bottom; bare surface was gold; bare crystal frequency, 6.011329 MHz.

[b] 0.5 mM MBPA in EtOH for ~ 24 h.

[c] 5 mM aq. Al(NO$_3$)$_3$ for ~ 4 h.

[d] 5 mM phosphoric acid for 4 h.

[e] Calf thymus DNA (3.8 mM NP) for 4 h.

[f] 0.24 mM Ru(phen)$_3^{2+}$ in MeCN for 4 h.

[g] The variation and drift of the signal over the series of measurements was about ± 1 Hz.

[h] Assuming roughness factor for gold of 2 (total surface area, 0.6 cm$^2$). For comparison, for a close-packed monolayer of MBPA, $\Gamma = 0.6 \times 10^{-9}$ mol/cm$^2$.

[i] Assuming an Al(H$_2$O)$_3^{3+}$ until adsorbed.

[j] Corresponding to moles of nucleotide phosphate (NP) per cm$^2$.

Clearly, the crystal frequency decreases as the Al$_2$(C$_4$BP) film forms and DNA and Ru(phen)$_3^{2+}$ are adsorbed on the surface, showing an increase of mass on the crystal during the different stages. The mass change, $\Delta m$, can be related to the

frequency chang , $\Delta f$, by the Sauerbrey equation:

$$\Delta m = -(A\sqrt{\rho_q \mu_q}/2F_0^2)\Delta f$$

where $F_0$ is the fundamental frequency of the unloaded crystal

(6 MHz), A is the electrode area (0.159 $cm^2$), $\rho_q$ is the density

5        of quartz (2.65 $g/cm^3$) and $\mu_q$ is the shear modulus of quartz

($2.95 \times 10^{11}$ $dyne/cm^2$). With these constants,

$$\Delta m \ (ng) = -1.95 \ \Delta f \ (Hz)$$

The mass changes calculated in this way are also given in

Table 1. These can be converted to approximate surface

10       concentrations, $\Gamma$, assuming a roughness factor of 2 (i.e.,

total surface area of both sides of the quartz crystal of 0.6

$cm^2$). ECL could also be detected from both gold surfaces of

the quartz crystal, when used as a substrate for $Al_2(C_4BP)$ film

formation, DNA adsorption and $Ru(phen)_3^{2+}$ association and then

15       scanned in the TPrA solution.

The electrode surface can be designed with

immobilized DNA without adsorbing a detector molecule,

$Ru(phen)_3^{2+}$. A ds-DNA on the surface can be detected by

electrogenerated chemiluminescence of associated $Ru(phen)_3^{2+}$.

20       Single-stranded DNA can also be immobilized on the $Al_2(C_4BP)$

film surface and then hybridized with complementary DNA in

solution with detection of the ds-DNA produced by ECL.

## Example 2

### Single-strand DNA immobilization and hybridization.

λ-1 tagged ss-DNA (i.e., labeled with $Ru(bpy)_3^{2+}$) was immobilized on an aluminum phosphate film of the present invention by immersing the film in the DNA solution (Fig. 4A). The amount of immobilized DNA-$Ru(bpy)_3^{2+}$ on the surface was determined by ECL resulting from the oxidation of $Ru(bpy)_3^{2+}$ and TPrA in a solution.

Untagged λ-1c ss-DNA was immobilized on an aluminum phosphate of the present invention. The λ-1c ss-DNA containing film was incubated in a complementary strand λ-1 tagged ss-DNA solution at 60°C for 5 min and then cooled to room temperature gradually; during this cooling the ss-DNA hybridized with the complementary strand DNA (Fig. 4B). The hybridized DNA-$Ru(bpy)_3^{2+}$ on the film was detected by ECL as described above.

Poly(dA) was immobilized on an aluminum phosphate film of the present invention by soaking the film in a poly(dA) solution. After the immobilization, poly(dT) was hybridized with poly(dA) to produce poly(dA)∘poly(dT) ds-DNA on the surface by incubating the film in a poly(dT) solution at 70°C for 5 min and then cooled to the room temperature gradually (Fig. 4C). To intercalate $Ru(phen)_3^{2+}$ into the ds-DNA (poly(dA)∘poly(dT)), the $Al_2(C_4BP)$/poly(dA)∘poly(dT) film was treated with a $Ru(phen)_3^{2+}$ solution. The hybridized poly(dA)∘poly(dT)-$Ru(phen)_3^{2+}$ on the surface was determined by ECL based on the oxidation of $Ru(phen)_3^{2+}$ and TPrA in the
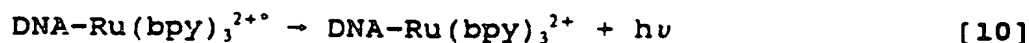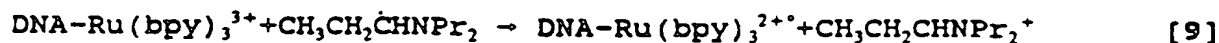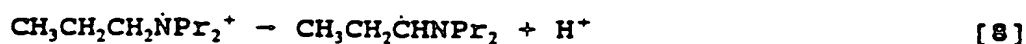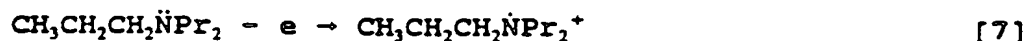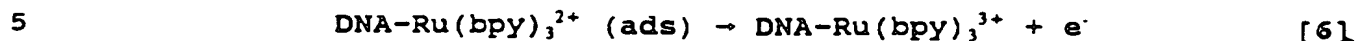
solution.

### Example 3

### Immobilization and Detection of 30 bp ss-DNA, Immobilized On Aluminum Phosphate Film.

5        The aluminum phosphate film, prepared as described
above was immersed in a 1.38 $\mu$M solution of $\lambda$-1 30 bp ss-DNA
(tagged with Ru(bpy)$_3^{2+}$) for ~2 h.  This was employed as a
working electrode for an ECL experiment in a 0.19 M phosphate
buffer (pH 7) containing 0.13 M TPrA.  Cyclic voltammograms

10       and emission transients were obtained by scanning the
potential of the electrode from 0 to 1.6 V vs, a saturated
calomel electrode (SCE).  A representative voltammogram and
emission detected with a single-photon-counting system are
shown in Figures 5A-5C.  The broad oxidation was at - 1.2 V

15       and a small reduction wave at -0.35 V observed in the
voltammogram (Fig. 5A) arise from the oxidation of TPrA,
Ru(bpy)$_3^{2+}$ and the Au substrate and the reduction of the oxide
of Au.  The ECL emission from the Al$_2$(C$_4$BP)/$\lambda$-1 ss-DNA-
Ru(bpy)$_3^{2+}$ electrode (Fig. 5B) demonstrates that the $\lambda$-1 30 bp

20       ss-DNA-Ru(bpy)$_3^{2+}$ was immobilized on the film.  The decay of
the light intensity (I) as a function of time (t) was also
investigated with the single-photon-counting system.  The
light intensity detected by the photomultiplier tube, when the
potential was stepped from 0 to 1.50 V, decreased with time

25       (Fig. 6).  The decay of intensity with time suggests
desorption of ss-DNA-Ru(bpy)$_3^{2+}$ from the electrode surface or

irreversible decomposition of the emitter. Emission results
from the energetic electron-transfer reaction between
electrogenerated $Ru(bpy)_3^{3+}$ and an intermediate in the
oxidation of TPrA:

5

$$DNA\text{-}Ru(bpy)_3^{2+} \ (ads) \rightarrow DNA\text{-}Ru(bpy)_3^{3+} + e^- \qquad [6]$$

$$CH_3CH_2CH_2\overset{.}{N}Pr_2 - e \rightarrow CH_3CH_2CH_2\overset{.}{N}Pr_2^+ \qquad [7]$$

$$CH_3CH_2CH_2\overset{.}{N}Pr_2^+ \rightarrow CH_3CH_2\overset{.}{C}HNPr_2 + H^+ \qquad [8]$$

$$DNA\text{-}Ru(bpy)_3^{3+}+CH_3CH_2\overset{.}{C}HNPr_2 \rightarrow DNA\text{-}Ru(bpy)_3^{2+\circ}+CH_3CH_2CHNPr_2^+ \qquad [9]$$

$$DNA\text{-}Ru(bpy)_3^{2+\circ} \rightarrow DNA\text{-}Ru(bpy)_3^{2+} + h\nu \qquad [10]$$

10      No ECL emission was observed from a film prepared by immersing
it in solution of unlabeled ss-DNA: 1.38 $\mu$M $\lambda$-1 30 bp ss-DNA
or a 0.37 $\mu$M $\lambda$-1c 30 bp ss-DNA solution (Fig. 5C).

A control experiment with the aluminum phosphate
film immersed in a 304 nM $Ru(bpy)_3^{2+}$ solution for - 4 h shows a
15      negligible ECL signal, indicating that $Ru(bpy)_3^{2+}$ does not
adsorb on the film and oxidize.

Example 4

Detection of 30 bp ss-DNA Hybridized to
Complementary ss-DNA Tagged With A Ruthenium Label.

20              After the immobilization of unlabeled $\lambda$-1c 30 bp ss-
DNA on the $Al_2(C_4BP)$ film, the film was immersed in a 1.38 $\mu$M
complementary strand ss-DNA (tagged with $Ru(bpy)_3^{2+}$) ($\lambda$-1 30 bp
ss-DNA-$Ru(bpy)_3^{2+}$) solution. The film in the solution was
gradually heated to 60°C in water bath, incubated at 60°C for

5 min. and then slowly cooled to room-temperature, during
which the λ-1 ss-DNA-Ru(bpy)$_3$$^{2+}$ was hybridized with the
complementary strand λ-1c ss-DNA on the surface as illustrated
in Fig. 4B.  The film with hybridized DNA was employed as the
5       working electrode in an ECL cell as described above.  The ECL
emission from the film was observed as shown in Fig. 7A.
However, when λ-1 ss-DNA, rather than λ-1c ss-DNA, was
immobilized on the film and followed by the same hybridization
procedure as described above by incubating the film in the λ-1
10      ss-DNA-Ru(bpy)$_3$$^{2+}$ solution, heating to 60°C, and then cooling
to room-temperature, no obvious ECL emission was evident as
shown in Fig. 7B.  This demonstrates that the ECL emission in
Fig. 7A arises from the hybridization of immobilized λ-1c ss-
DNA with λ-1 ss-DNA-Ru(bpy)$_3$$^{2+}$ in the solution.  The emission
15      again results from the oxidation of Ru(bpy)$_3$$^{2+}$-tagged
hybridized ss-DNA and TPrA in the solution as described by
Eqs. [6] - [10].  In this experiment the Al$_2$(C$_4$BP) film should
be incubated in the λ-1 ss-DNA solution for a sufficiently
long time (at least 4 h) to cover all possible Al (III)
20      binding sites on the film with the λ-1 ss-DNA before the
Al$_2$(C$_4$BP)/λ-1 ss-DNA electrode is exposed to the λ-1 ss-DNA-
Ru(bpy)$_3$$^{2+}$.  It is preferred to cover all Al$^{3+}$ adsorption sites
to avoid emission from the film/λ-1 ss-DNA/λ-1 ss-DNA-
Ru(bpy)$_3$$^{2+}$ arising from some immobilization of ss-DNA-
25      Ru(bpy)$_3$$^{2+}$.

## Example 5

## Detection of poly(dA) Hybridized to poly(dT) using a Ruthenium Label.

The aluminum phosphonate film, prepared as described

5    above, was immersed in a 21 $\mu$M poly(dA) solution for ~4 h,

then in a 0.24 mM Ru(phen)$_3^{2+}$ solution for ~4 h.  When the

Al$_2$(C$_4$BP)/poly(dA)/Ru(phen)$_3^{2+}$ film was employed as a working

electrode in a solution of 0.19 M phosphate buffer (pH 7)

containing 0.13 M TPrA, no ECL emission was observed (Fig.

10   8A).  This is consistent with the lack of association of

Ru(phen)$_3^{2+}$ with ss-DNA.  However, a film of poly(dA)

hybridized with only poly(dT) to form ds-DNA did produce ECL.

This film, formed by immersion in the poly(dA) solution, was

incubated in a 38 $\mu$M poly(dT) solution (gradually heated to

15   70°C in a water bath, incubated at 70°C for 5 min and then

slowly cooled to room temperature).  It was then treated with

a 0.24 mM Ru(phen)$_3^{2+}$ aqueous solution and used as a working

electrode in the TPrA-containing phosphate buffer for ECL

investigation.  ECL emission was observed as shown in a

20   representative emission transient (Fig. 8B), demonstrating

that poly(dT) in the solution hybridized with the poly(dA) on

the surface to produce a ds-DNA [poly(dA)°poly(dT] and

Ru(phen)$_3^{2+}$ intercalated with the ds-DNA as shown in Fig. 4c.

These examples show that Ru(phen)$_3^{2+}$ only

25   intercalates with ds-DNA [poly(dA)°poly(dT], but not with ss-

DNA (poly(dA)).  Intercalation of Ru(phen)$_3^{2+}$ with ss-DNA and

ds-DNA, ECL emission was m asured from a 43 nM Ru(phen)$_3^{2+}$

solution, a 43 nM $Ru(phen)_3^{2+}$ containing 57 $\mu$M nucleotide
phosphate ([NP]) of calf thymus ss-DNA and a 43 nM $Ru(phen)_3^{2+}$
containing 33 $\mu$M [NP] of calf thymus ds-DNA with a QPCR
analyzer.  Results are given in Table 2,

## Table 2

### ECL of Solutions of 43 nM Ru(phen)$_3^{2+}$

### with ss-DNA and ds-DNA

| Solution | I Ru(phen)$_3^{2+}$ alone | | II[a] ss-DNA+Ru(phen)$_3^{2+}$ | III[b] ds-DNA+Ru(phen)$_3^{2+}$ |
|---|---|---|---|---|
| Conc. NP($\mu$M) | | 0 | 57 | 33 |
| ECL(cts/s) | | 10068$\pm$23 | 10052$\pm$12 | 7779$\pm$268 |

[a] Ratio of moles of nucleotide phosphate (NP) of ss-DNA to

Ru(phen)$_3^{2+}$ = 1.3 x 10$^4$

[b] Ratio of moles of nucleotide phosphate (NP) of ds-DNA to

Ru(phen)$_3^{2+}$ = 7.7 x 10$^3$

indicating that the ECL emission decreased after ds-DNA was
added to the Ru(phen)$_3^{2+}$ solution.  A negligible change,
however, in the ECL signal was shown when ss-DNA was added,
further demonstrating that Ru(phen)$_3^{2+}$ only intercalates with
ds-DNA, but not with ss-DNA.

A control experiment in which a film, after the
immobilization of poly(dA), was incubated in a 21 $\mu$M poly(dA)
or a 46 $\mu$M poly(dC) solution instead of poly(dT) (70°C for 5
min then cooled to room-temperature) and then treated with
Ru(phen)$_3^{2+}$ for ~2 h produces no ECL emission.  This indicates
that the Al$_2$(C$_4$BP)/poly(dA) electrode can distinguish a
complementary strand of poly(dT) DNA from non-complementary
ones.

## Example 6

TEM Images of the film and calf thymus ds-DNA.

Samples, prepared by coating Au on a Formvar film on
a #400 Cu grid with a vacuum evaporator, fabricating the
5    aluminum phosphate film on the Au as described above, and then
immobilizing DNA on the $Al_2(C_4BP)$ film by immersing the film in
a 1.65 mM [NP] of calf thymus ds-DNA for 4 h, were imaged with
a transmission electron microscope (TEM) at 80 KV.  As shown
in Figs. 9A-9C the featureless Au substrate (Fig. 9A) shows
10   formation of crystalline islands of $Al_2(C_4BP)$ film (Fig. 9B)
and clumps of DNA (Fig. 9C).  The film (Fig. 10A) was then
treated with either 1.65 mM [NP] calf thymus ds-DNA (Fig. 10B)
or an identical solution of ds-DNA that had been subjected to
sonication for 6 h (Fig. 10C).  The results indicate that
15   smaller clumps of DNA are adsorbed on the film after
sonication.

A further embodiment of the invention is shown in
Figs. 11A and 11B.  A sensor surface having a multilayer film
with bonding groups is provided with a complete set of
20   oligonucleotide probes using similar techniques described
above for adsorbing ds-DNA and for SS-DNA.  See Fig. 11A.  The
sensor surface of Fig. 11A is contacted with DNA to be
sequenced.  The above disclosed ECL procedures are then used
to recognize zones with a complementary sequence.  (Fig. 11B).

25   When chips are used, different types of ss-DNA that
make up a test sequnce are attached to the surface of a chip
to make an array.  The chip array is then exposed to the

sample solution to be sequenced, with formation of ds-DNA at
the appropriate location being recognized by the ECL approach.

Although the invention has been described in
conjunction with the specific embodiments, it is evident that
5    many alternatives and variations will be apparent to those
skilled in the art in light of the foregoing description.
Accordingly, the invention is intended to embrace all of the
alternatives and variations that fall within the spirit and
scope of the appended claims.

SEQUENCE LISTING

(1)  GENERAL INFORMATION:

    (i)    APPLICANTS:  Allen J. Bard and Xia-Hong Hu

   (ii)    TITLE OF THE INVENTION: BIOSENSOR FOR AND
        METHOD OF ELECTROGENERATED CHEMILUMINESCENT
        DETECTION OF NUCLEIC ACID ADSORBED TO A SOLID
        SURFACE

  (iii)    NUMBER OF SEQUENCES: 3

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    (v)    COMPUTER READABLE FORM:
        (A)    MEDIUM TYPE: Diskette, 3.50 inch, 1.44 Mb
            storage
        (B)    COMPUTER: IBM COMPATIBLE
        (C)    OPERATING SYSTEM: MS-DOS 5.0
        (D)    SOFTWARE: WordPerfect 5.1

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  (vii)    PRIOR APPLICATION DATA: N/A

 (viii)    ATTORNEY/AGENT INFORMATION:
        (A)    NAME: Michael S. Marcus
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        (B)    TELEFAX: (202) 857-7929


(2)  INFORMATION FOR SEQ ID NO: 1:

    (i)    SEQUENCE CHARACTERISTICS:
        (A)    LENGTH: 30
        (B)    TYPE: nucleic acid
        (C)    STRANDEDNESS: single
        (D)    TOPOLOGY: linear

    (ii)               MOLECULE TYPE:

  (iii)     HYPOTHETICAL: NO

   (iv)     ANTI-SENSE: NO

    (v)     FRAGMENT TYPE: LAMBDA

   (vi)     ORIGINAL SOURCE:
            (A)   ORGANISM:
            (B)   STRAIN:
            (C)   INDIVIDUAL ISOLATE:
            (D)   DEVELOPMENTAL STAGE:
            (E)   HAPLOTYPE:
            (F)   TISSUE TYPE:
            (G)   CELL TYPE:
            (H)   CELL LINE:
            (I)   ORGANELLE:

  (vii)   IMMEDIATE SOURCE:
            (A)   LIBRARY:
            (B)   CLONE:

 (viii)   POSITION IN GENOME:
            (A)   CHROMOSOME SEGMENT: N/A
            (B)   MAP POSITION: N/A
            (C)   UNITS: N/A

   (ix)    FEATURE:
            (A)   NAME/KEY:
            (B)   LOCATION: N/A
            (C)   IDENTIFICATION METHOD:
            (D)   OTHER INFORMATION: N/A

    (x)    PUBLICATION INFORMATION:
            (A)   AUTHORS:
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            (K)   RELEVANT RESIDUES In SEQ ID NO: 1:

   (xi)    SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GAAAATGTGC TGACCGGACA TGAAAATGAG 30

(2)  INFORMATION FOR SEQ ID NO: 2:

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 (i)    SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 30
        (B)   TYPE:
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

(ii)    MOLECULE TYPE:

(iii)   HYPOTHETICAL: NO

(iv)    ANTI-SENSE: N/A

 (v)    FRAGMENT TYPE: LAMBDA

(vi)    ORIGINAL SOURCE:
        (A)   ORGANISM:
        (B)   STRAIN:
        (C)   INDIVIDUAL ISOLATE:
        (D)   DEVELOPMENTAL STAGE:
        (E)   HAPLOTYPE:
        (F)   TISSUE TYPE:
        (G)   CELL TYPE:
        (H)   CELL LINE:
        (I)   ORGANELLE:

(vii)   IMMEDIATE SOURCE:
        (A)   LIBRARY:
        (B)   CLONE:

(viii)  POSITION IN GENOME:
        (A)   CHROMOSOME SEGMENT: N/A
        (B)   MAP POSITION: N/A
        (C)   UNITS: N/A

(ix)    FEATURE:
        (A)   NAME/KEY:
        (B)   LOCATION: N/A
        (C)   IDENTIFICATION METHOD:
        (D)   OTHER INFORMATION: N/A

(x)     PUBLICATION INFORMATION:
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        (G)   DATE:
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        (J)   PUBLICATION DATE:
        (K)   RELEVANT RESIDUES In SEQ ID NO: 2:
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CTCATTTTCA TGTCCGGTCA GCACATTTTC 30

CLAIMS

1.       A method of detecting nucleic acids comprising:

    a.    forming a sensor surface bearing a film having
          metallic centers;

    b.    adsorbing at least one nucleic acid onto to
5         said metal centers;

    c.    reacting nucleic acid adsorbed to said film
          with a luminescent metal label; and

    d.    detecting the nucleic acid metal label chelates
          formed in step (c) via electrogenerated
10        chemiluminescence of said chelates.


2.       The method of claim 1, wherein the nucleic acid of
step (b) is double-stranded.


3.       The method of claim 1, wherein the nucleic acid of
step (b) is single-stranded.


4.       The method of claim 3, wherein step (c) further
comprises binding the single-stranded nucleic acid adsorbed to
the film, to a complementary single-stranded nucleic acid
sequence.


5.       The method of claim 1, wherein said electrogenerated
chemiluminescence arises from a reaction of an
electrogenerated metal label in the nucleic acid -metal label

chelate and a suitable coreactant.


6.        The method of claim 5, wherein said electrogenerated
metal label is produced via emission from the sensor which is
an electrode.


7.        A method of detecting nucleic acids comprising:

          a.    forming an electrode by adsorbing at least one
nucleic acid onto said electrode;

          b.    reacting nucleic acid adsorbed to said
electrode with a luminescent label; and

          c.    detecting the nucleic acid label formed in step
(b) via electrogenerated chemiluminescence.


8.        The method of claim 7, wherein the nucleic acid of
step (b) is double-stranded.


9.        The method of claim 7, wherein the nucleic acid of
step (b) is single-stranded.


10.       The method of claim 8, wherein step (c) further
comprises binding the single-stranded nucleic acid adsorbed to
the electrode, to a complementary single-stranded nucleic acid
sequence.


11.       The method of claim 7, wherein said electrogenerated
chemiluminescence arises from a reaction of an

electrogenerated metal label in the nucleic acid - metal label
chelate and a suitable coreactant.

12.      The method of claim 11, wherein said
electrogenerated metal label is produced via emission from the
electrode.

13.      An aluminum alkanebisphosphonate biosensor
comprising:
         a substrate and a first metallic layer; and
5        an aluminum alkanebisphosphonate layer forming (Al)
         metallic centers.

14.      A biosensor according to claim 13, wherein said
biosensor further includes single-strand DNA immobilized to
said aluminum centers.

15.      A biosensor according to claim 14, wherein said DNA
is labelled with and an osmium or ruthenium moiety.

16.      A biosensor according to claim 13, wherein said
biosensor further includes double-strand DNA immobilized to
said aluminum centers.

17.      A biosensor according to claim 16, wherein said DNA
is labelled with an osmium or ruthenium moiety.

18.        A method of preparing a biosensor for nucleic acid,
comprising:

          a)    treating a silicon wafer to form a chromium
layer and juxtaposed gold layer;

5          b)    contacting said layered wafer with an anchoring
agent; and

          c)    immersing the product of step b) alternately in
Al(NO$_3$)$_3$, bisphosphonic (H$_2$O$_3$P(CH$_2$)$_4$PO$_3$H$_2$) and Al(NO$_3$)$_3$ aqueous
solutions.


19.        A biosensor comprising:
          an electrode substrate, adsorbed ss-DNA or ds-DNA
immobilized on said electrode, and a detectable label on said
ss-DNA or ds-DNA.


20.        A biosensor according to claim 19, wherein said DNA
is labelled with an osmium or ruthenium moiety or a
luminescent label.

$$\xrightarrow[\text{EtOH}]{HS(CH_2)_4PO_3H_2}$$

Si Au

$$PO_3^{2-}$$

$$PO_3^{2-}$$

$$\xrightarrow[H_2O]{Al^{3+}}$$

$$PO_3 \diagdown \diagup$$

$$-Al-$$

$$PO_3 \diagup$$

$$\xrightarrow[H_2O]{H_2O_3P(CH_2)_4PO_3H_2}$$

$$PO_3 \diagdown \diagup O_3P \diagup\!\!\diagup PO_3^{2-}$$

$$-Al-$$

$$PO_3 \diagup \diagdown O_3P \diagup\!\!\diagup PO_3^{2-}$$

$$\xrightarrow[H_2O]{Al^{3+}}$$

$$PO_3 \diagdown \diagup O_3P \diagup\!\!\diagup PO_3 \diagdown \diagup$$

$$-Al- \qquad\qquad -Al-$$

$$PO_3 \diagup \diagdown O_3P \diagup\!\!\diagup PO_3 \diagup\diagdown$$

FIG. 1

FIG. 2

FIG. 3A

FIG. 3B

FIG. 3C

FIG. 4A

FIG. 4B

FIG. 4C

  
FIG. 5A

FIG. 5B

FIG. 5C

E (V) vs SCE

FIG. 6

FIG. 7A

FIG. 7B

EMISSION INTENSITY

5 kc/s

200 c/s

E (V) vs SCE

1.6        1.2        0.8        0.4        0

FIG. 8A

100 c/s

FIG. 8B

200 c/s

EMISSION INTENSITY

E (V) vs SCE

FIG. 9A

712 mm
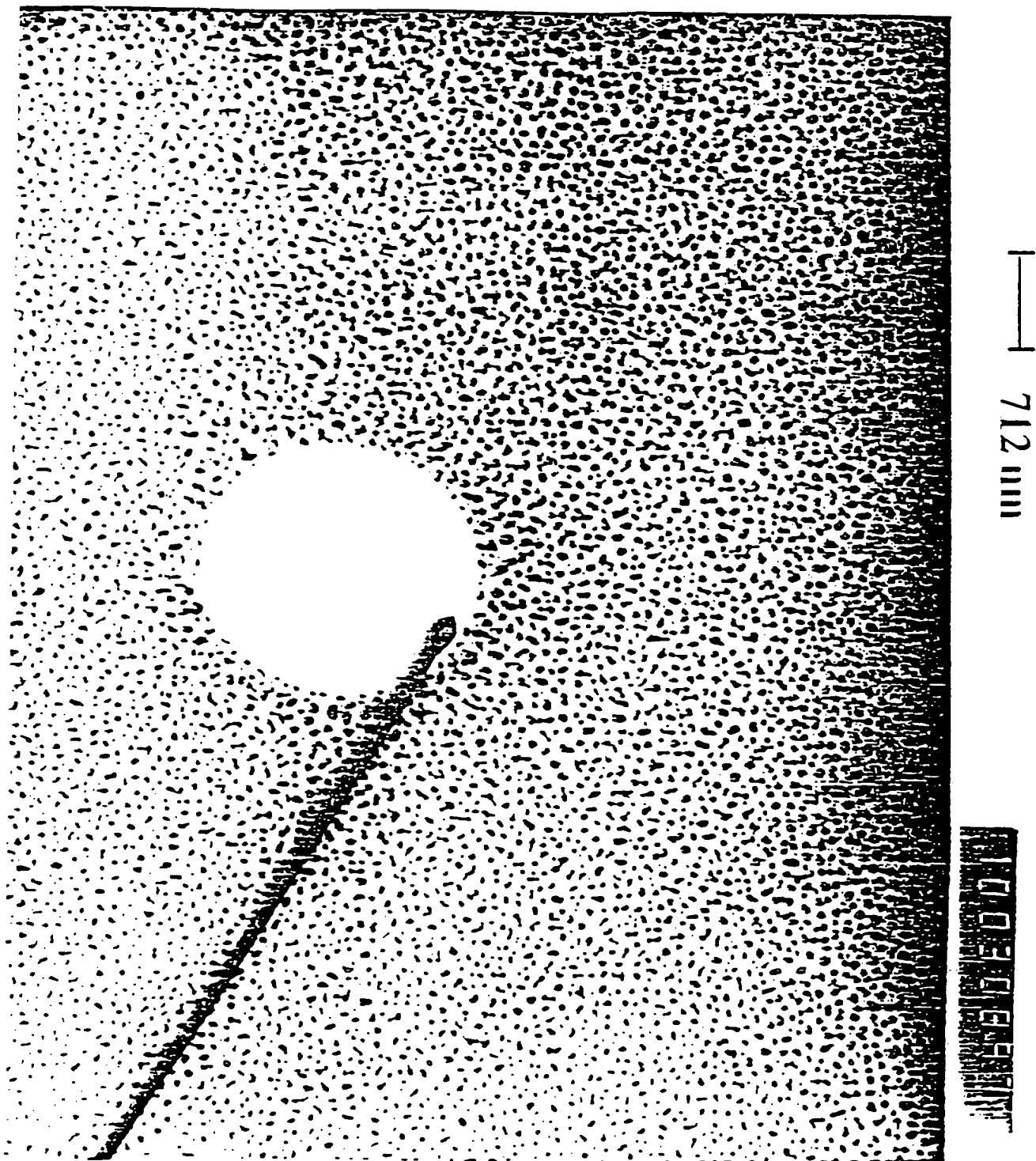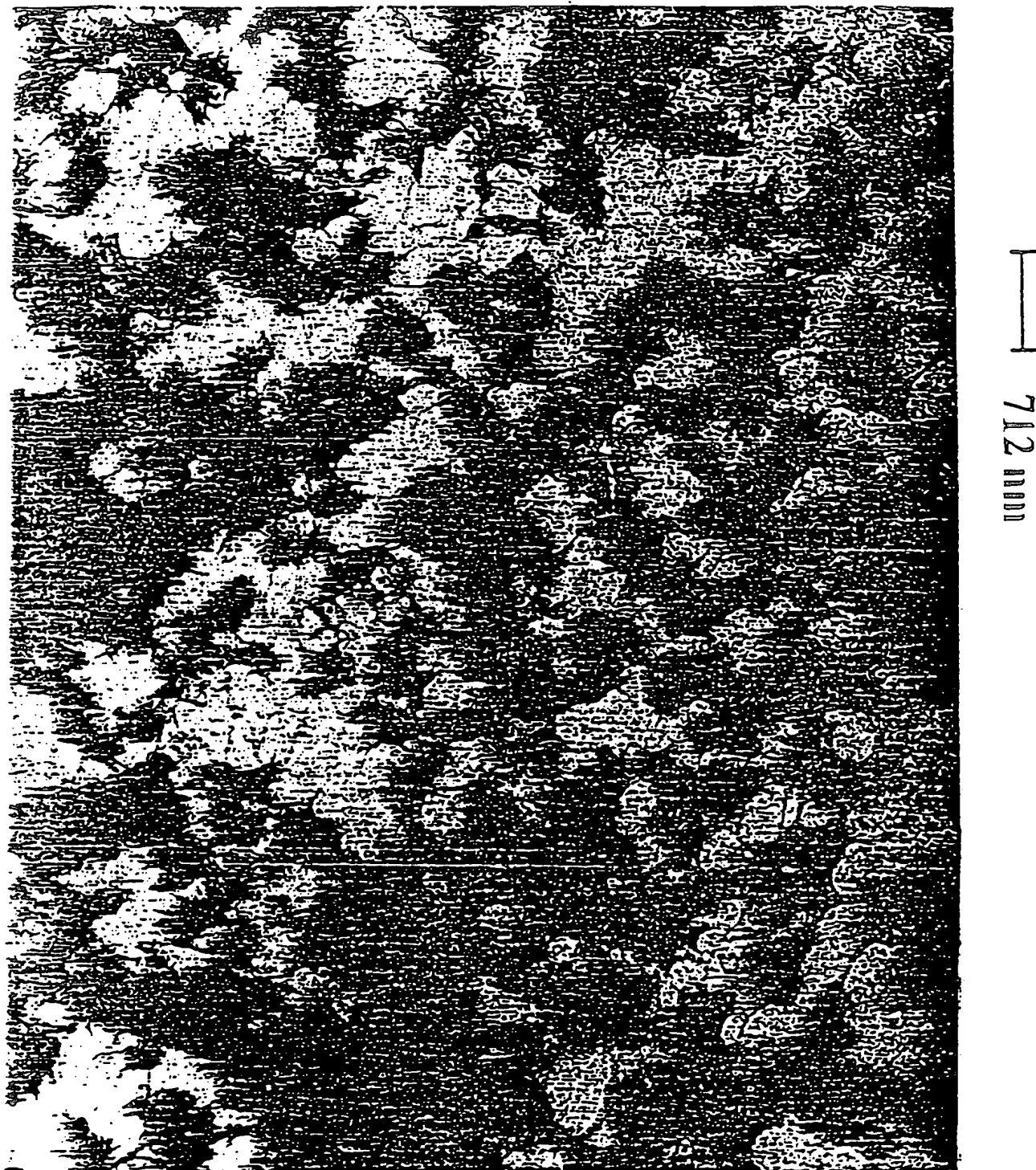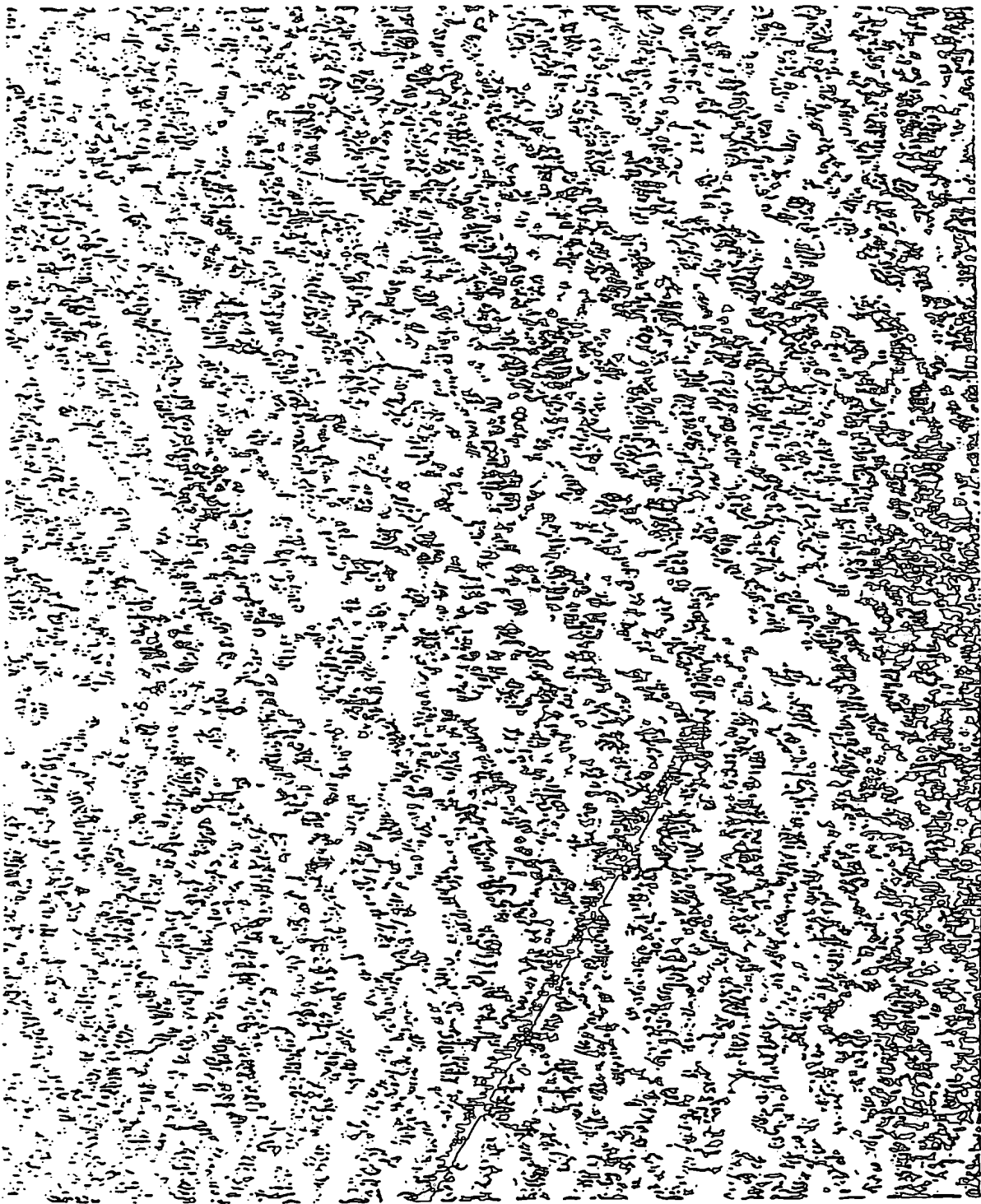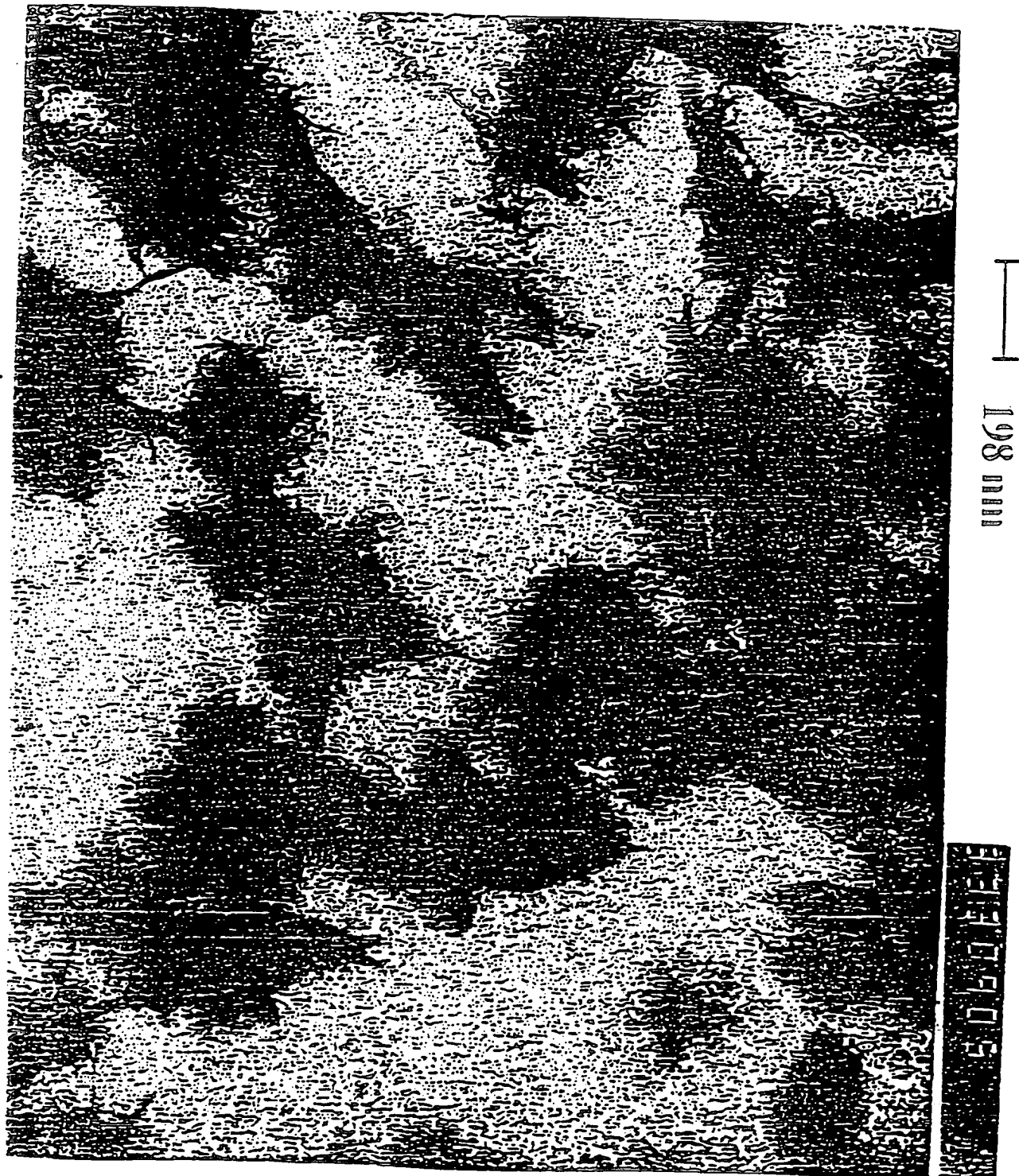
FIG. 9B

FIG. 9C

712 mm
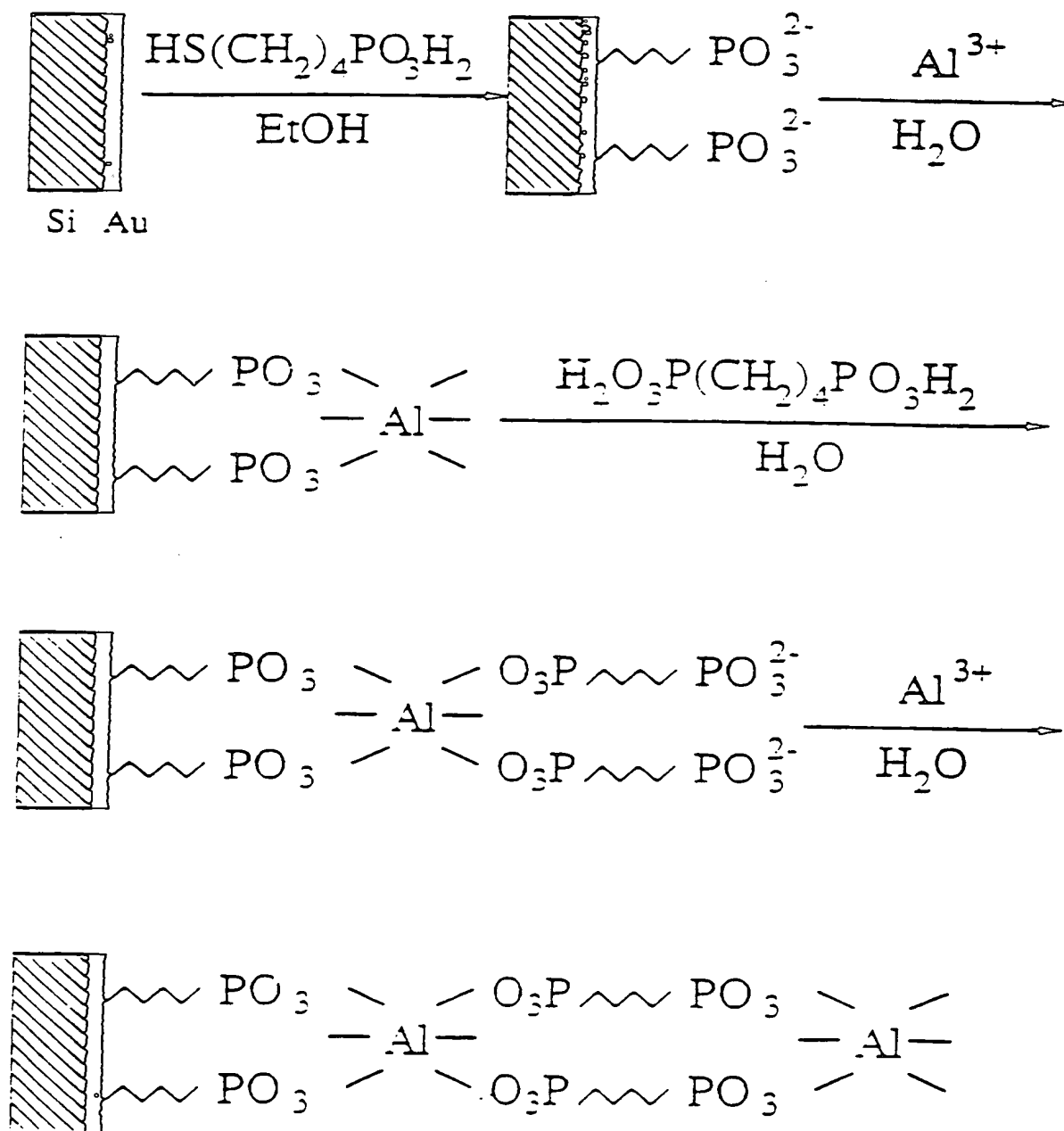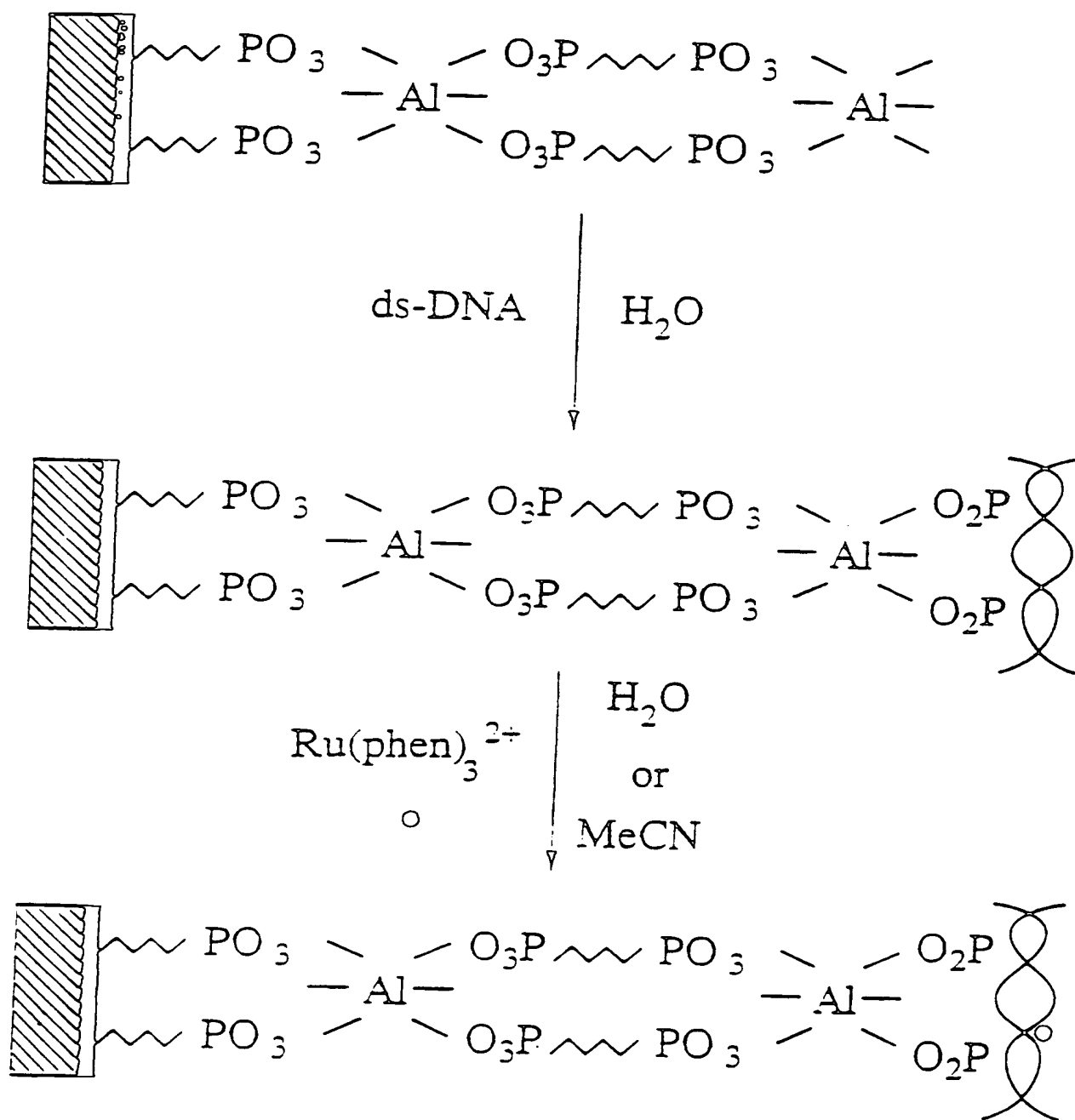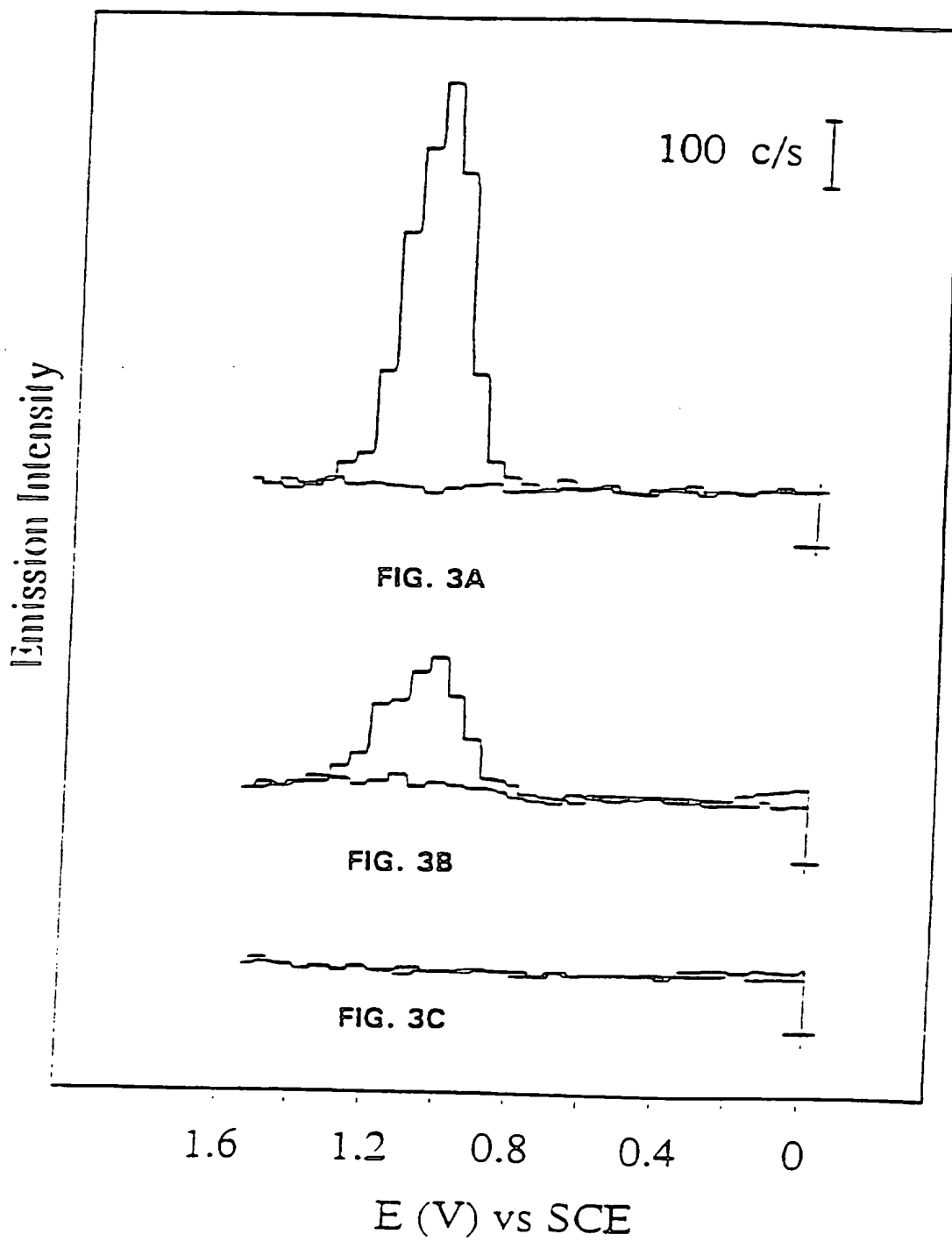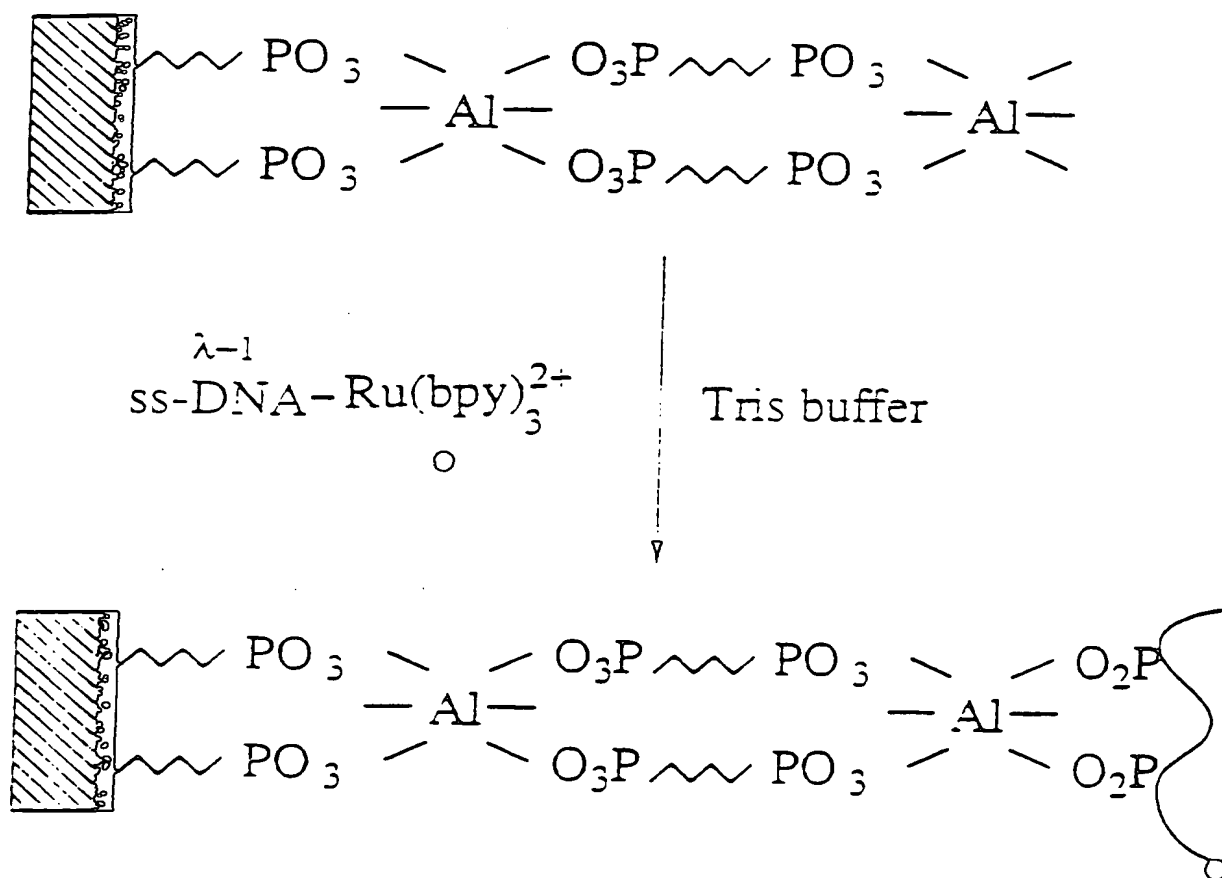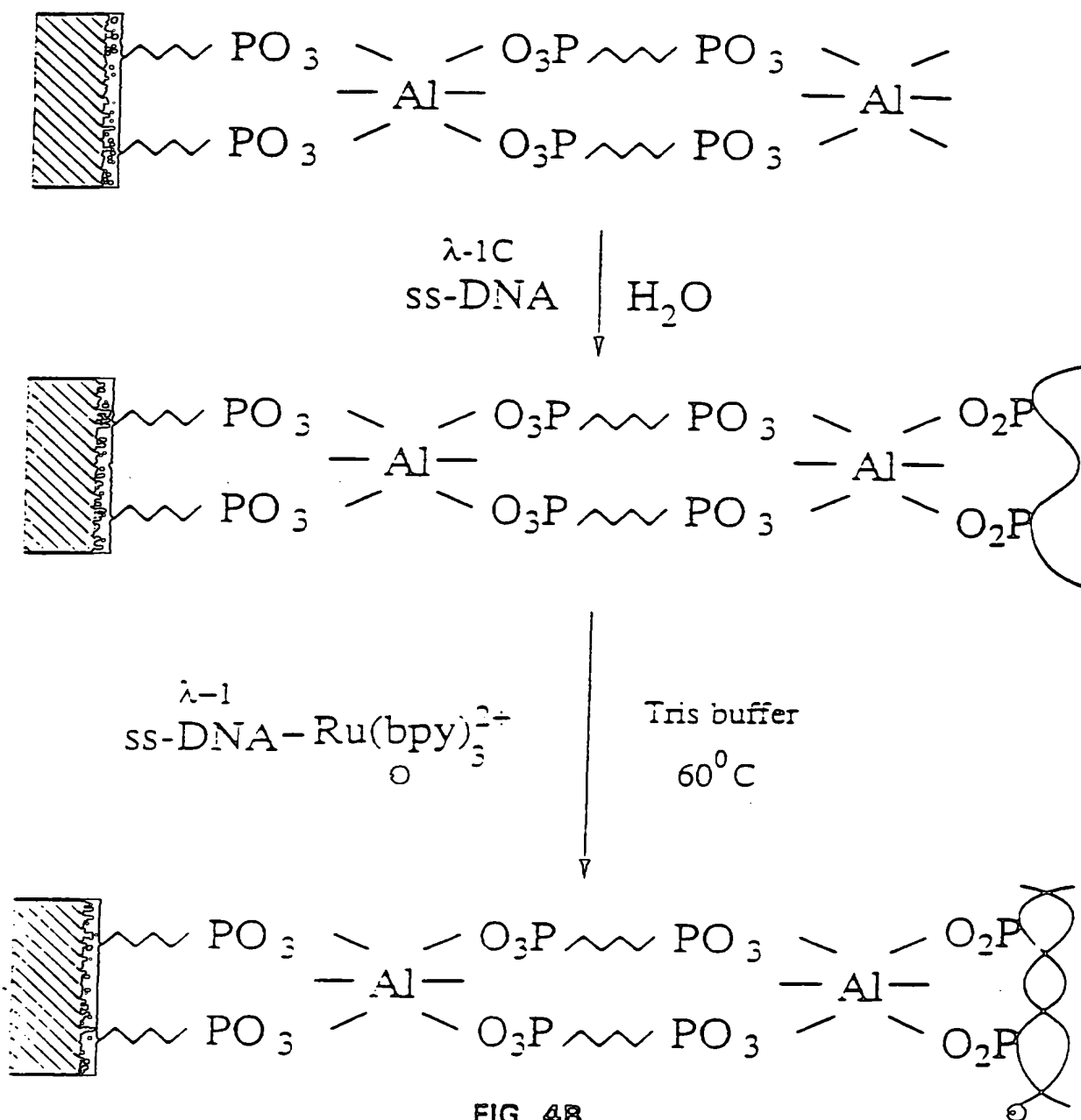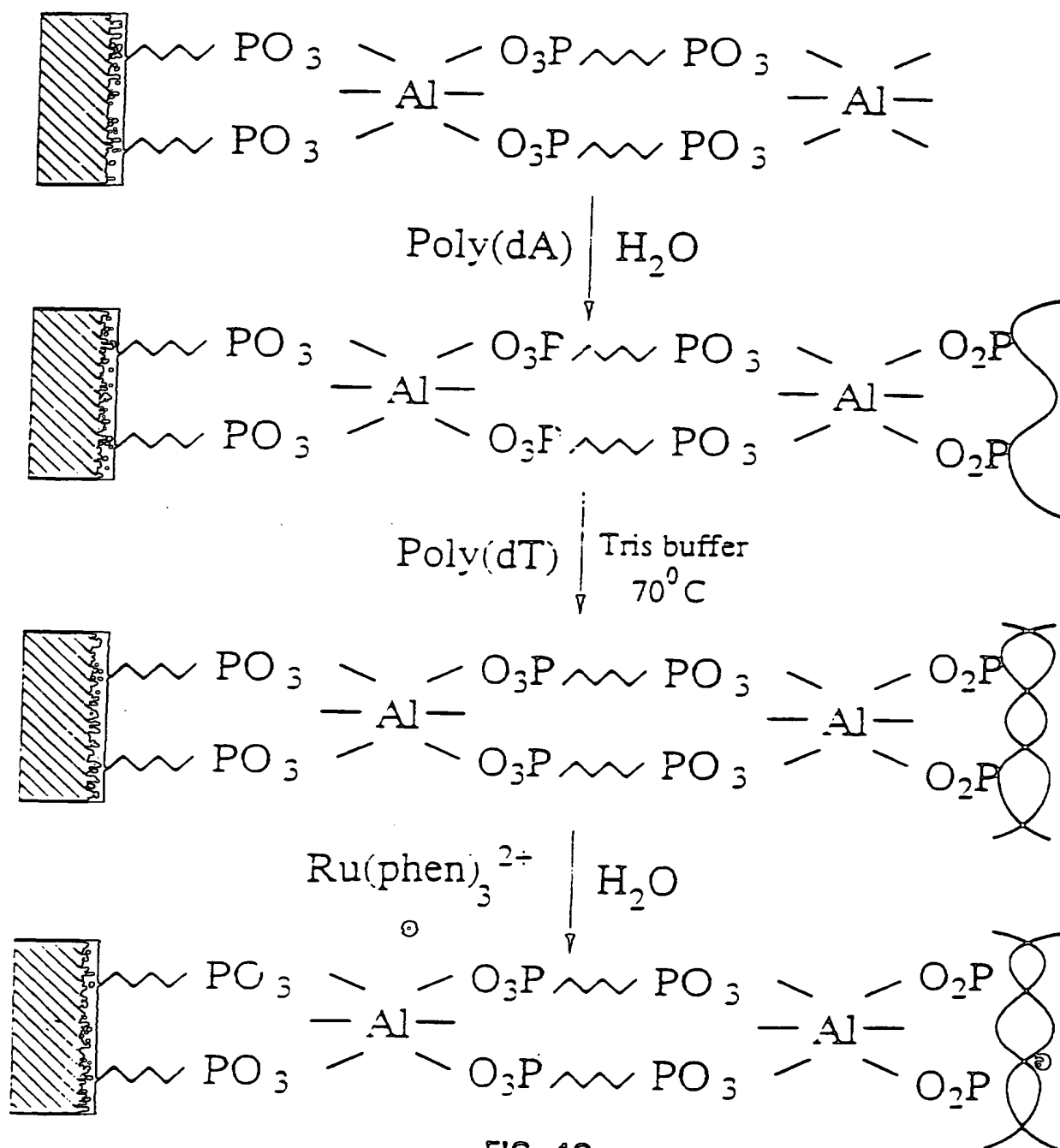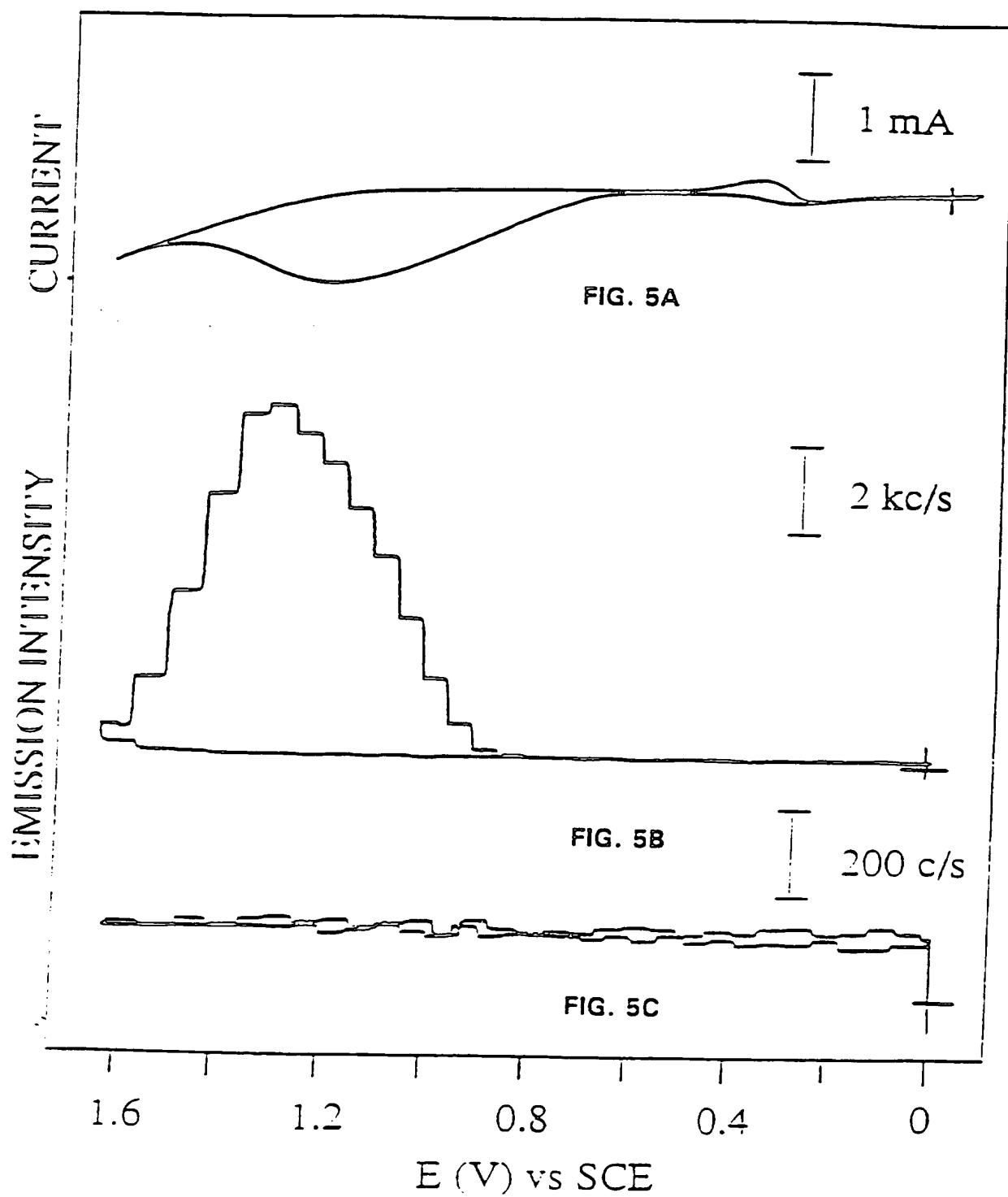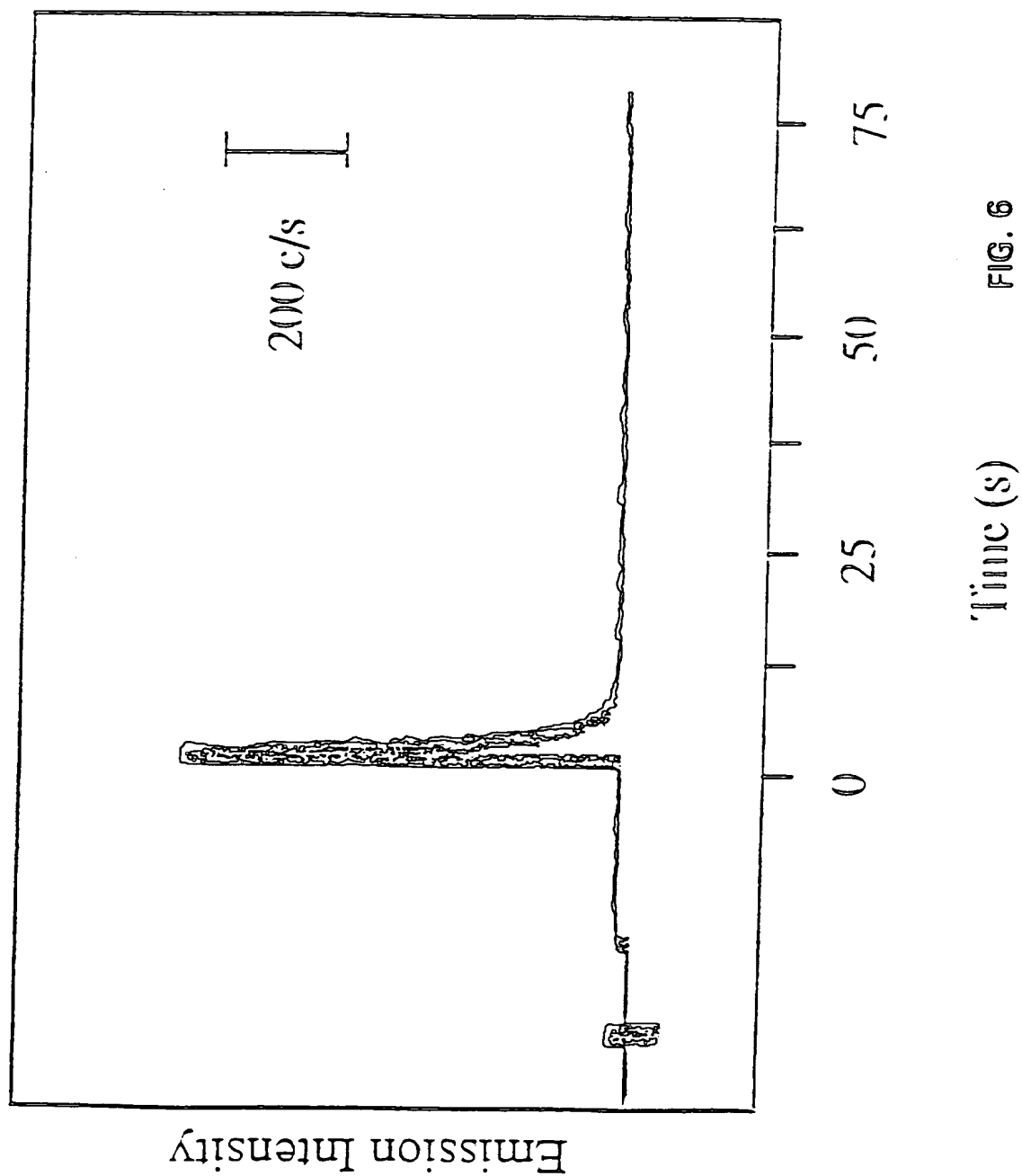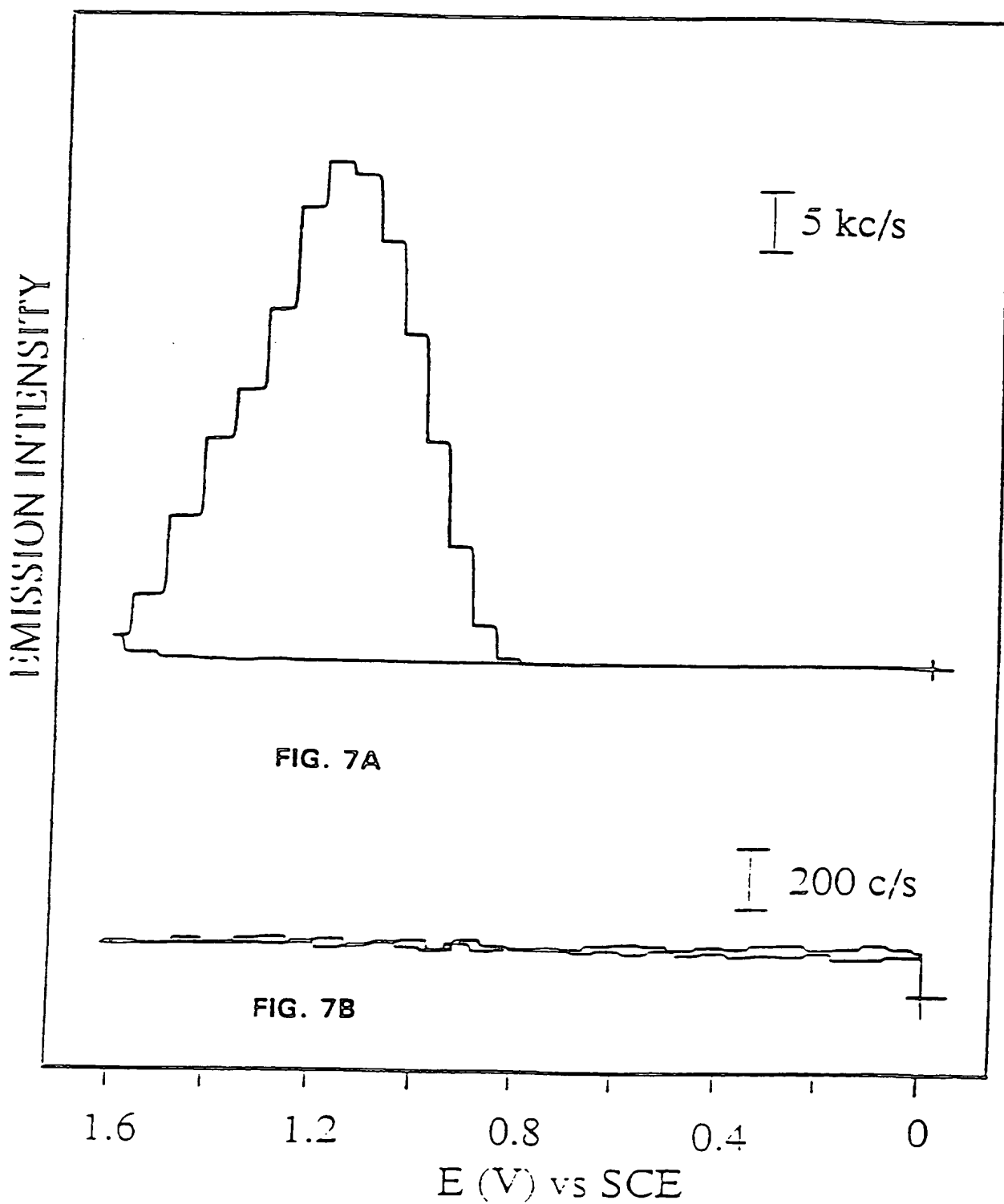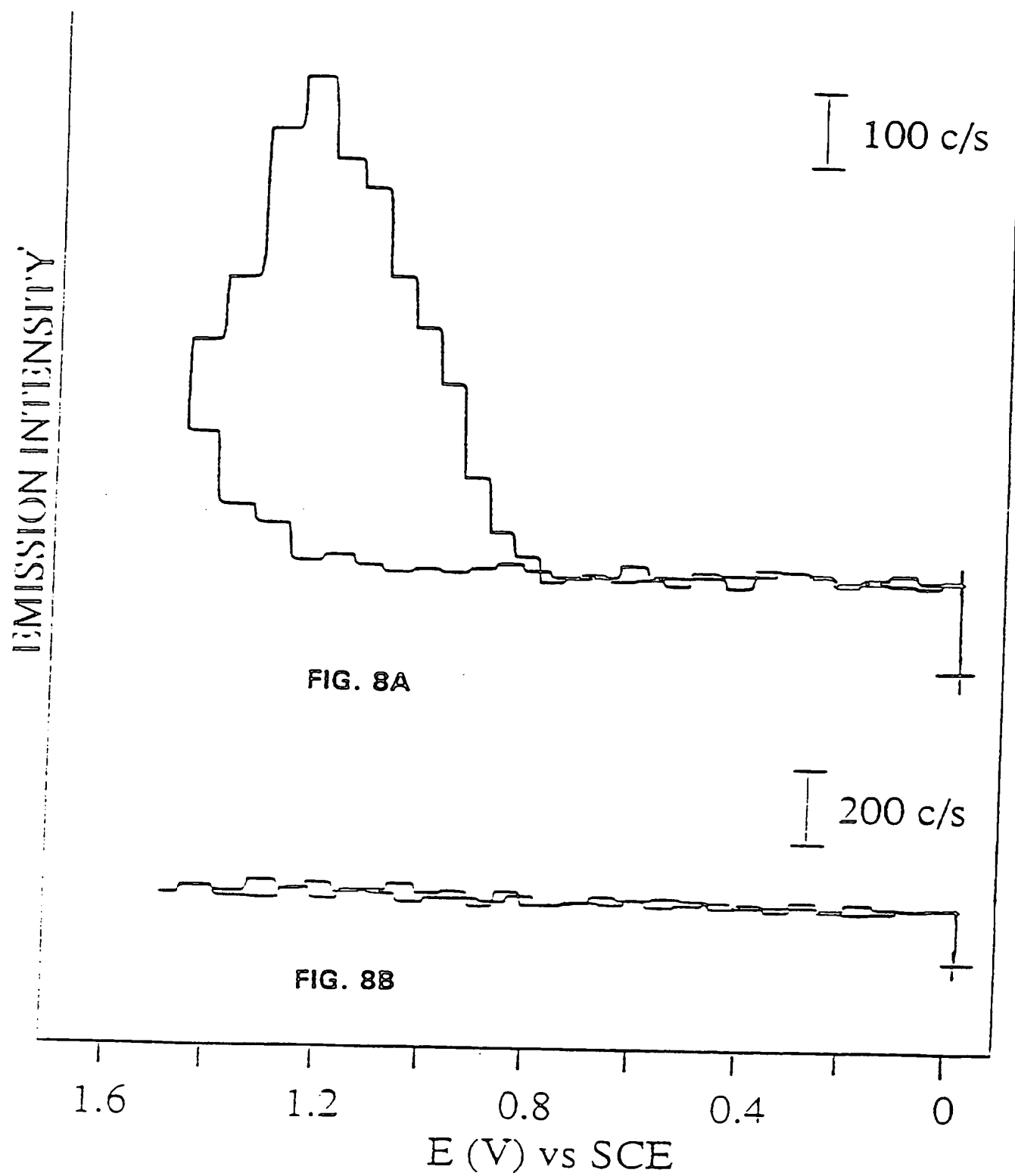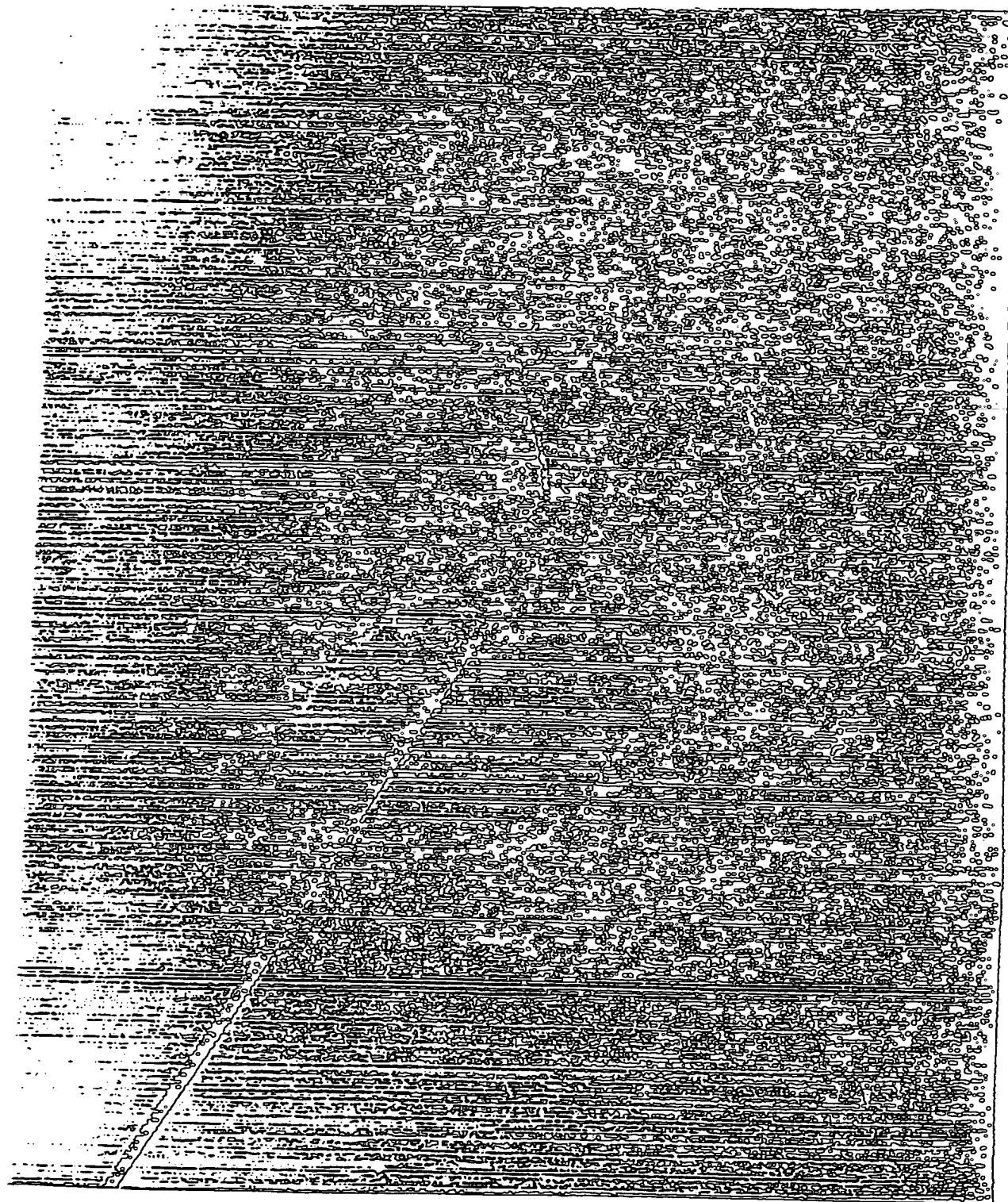
FIG. 10A

FIG. 10B

FIG. 10C

## SEQUENCING VIA ARRAY HYBRIDIZATION

1. Form array by surface attachment of complete set of olignucleotide probes (as above)



| AAAA... | AAAG... | AAAT... | AAAC... |

FIG    11A

2. Expose to DNA to be sequenced. Detect via ECL as above to recognize zones with complementary sequence.



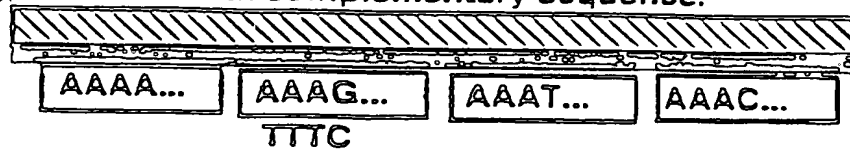| AAAA... | AAAG... | AAAT... | AAAC... |

TTTC

FIG. 11B

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6)  :Please See Extra Sheet.

US CL  :435/ 4, 5, 6, 7.1, 91.2; 536/24.3; 205/175;

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S.  :  435/ 4, 5, 6, 7.1, 91.2; 536/24.3; 205/175;

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | Analytical Chemistry, Volume 66, Number 6, issued 15 March 1994 , H. Su et al, "Interfacial Nucleic Acid Hybridization Studied by Random Primer 32P Labeling and Liquid-Phase Acoustic Network Analysis", pages 769-77, see Figure 2 and page 771, column 2, paragraph 3 and 4. | 19 |
| Y | WO 93/22678 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 11 November 1993, pages 3-5. | 1-20 |
| Y | US, A, 5,238,808 (BARD ET AL) 24 August 1993, column 5, lines 59-70, column 6. | 1-20 |
| Y | US, A, 3,591,838 (FUJIWARA ET AL) 06 July 1971, column 1, lines 48-58 . | 13-18 |

[X] Further documents are listed in the continuation of Box C.    [ ] See patent family annex.

| | |
|---|---|
| * | Special categories of cited documents: |
| "A" | document defining the general state of the art which is not considered to be of particular relevance |
| "E" | earlier document published on or after the international filing date |
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| "O" | document referring to an oral disclosure, use, exhibition or other means |
| "P" | document published prior to the international filing date but later than the priority date claimed |

| | |
|---|---|
| "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "&" | document member of the same patent family |

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 02 OCTOBER 1995 | 26OCT1995 |

| Name and mailing address of the ISA/US | Authorized officer |
|---|---|
| Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 | DIANNE REES |
| Facsimile No.  (703) 305-3230 | Telephone No.  (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US, A, 5,124,022 (EVANS, II ET AL) 23 June 1992, column 5, lines 48-59. | 1-20 |
| A | US, A, 5019,343 (HWA ET AL) 28 May 1991, column 3, lines 1-32. | 1-20 |

Form PCT/ISA/210 (continuation of second sheet)(July 1992)°

**A. CLASSIFICATION OF SUBJECT MATTER:**

IPC (6):

C12Q 1/00, 1/70, 1/68; C 12 P 19/34; C07H 21/04; C25D 11/12; GO1N 33/53

**B. FIELDS SEARCHED**

Electronic data bases consulted (Name of data base and where practicable terms used):

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APS, AIDSLINE, ANABSTR, AQUASCI, BIOBUSINESS, BIOSIS, BIOTECHABS, BIOTECHDS, CAPLUS, CEABA, EMBASE, JICST-EPLUS, LIFESCI, MEDLINE, PHIN, PROMT, SCISEARCH, TOXLINE,TOXLIT,USPATFULL,WPIDS

Search terms: biosensor, electrogenerated, ecl, electrochemiluminescence, alkanebisphosphonate, electrodes, chelators, polydentate, osmium or ruthenium labels,nucleic acid hybridization, immunoassays.